Monographs on Biochemistry

THE NATURE OF ENZYME ACTION

BY

W. M. BAYLISS, D.Sc., F.R.S.


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MONOGRAPHS ON BIOCHEMISTRY

EDITED BY

R. H. A. PLIMMER, D.Sc.

AND

F. G. HOPKINS, M.A., M.B., D.Sc., F.R.S.

GENERAL PREFACE.

THE subject of Physiological Chemistry, or Biochemistry, is enlarging its borders to such an extent at the present time that no single text-book upon the subject, without being cumbrous, can adequately deal with it as a whole, so as to give both a general and a detailed account of its present position. It is, moreover, difficult, in the case of the larger text-books, to keep abreast of so rapidly growing a science by means of new editions, and such volumes are therefore issued when much of their contents has become obsolete.

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R. H. A. P.
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BY

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PREFACE TO THIRD EDITION.

A CONSIDERABLE amount of new matter has been added to this edition, and the continued discovery of further facts has rendered it necessary to revise certain paragraphs. At the same time, it has been possible to abbreviate the description of some other work which has shown itself unable to stand the test of renewed investigation.

Attention is particularly directed to the chapters on "Reversibility" and on the "Combination" between enzyme and substrate, as also to the section on anti-enzymes, which have, for the most part, been rewritten. It will be found that many important new discoveries have been made in these fields. The facts referred to have made some modifications necessary in the point of view taken in previous editions; but, for the most part, these are in the direction of simplification. It is satisfactory to note that the doctrine of the importance of surface phenomena, or adsorption, in the action of enzymes, advocated in these pages from the first, is becoming more and more widely recognised.

The use of the word "Nature" in the title of this monograph has been subjected to criticism. It is said that the "Nature of Enzyme Action" is not explained. My object is to compare the various activities grouped under the head of "enzymic" with those known as "catalytic". If it is found that their nature is the same, a group of facts is brought under the application of more general laws, and, as I understand it, that is what "explanation" in Science essentially is.

W. M. B.

UNIVERSITY COLLEGE, LONDON,
July, 1913.

PREFACE TO SECOND EDITION.

The large amount of work on enzymes published in the short space of time since the first edition of this monograph was written necessitates the addition of much new matter.

The number of references in the Bibliography, in accordance with this increase in knowledge, has considerably increased.

In a few places some amplification has been thought desirable in order to render the meaning easier to understand.

Headings of paragraphs have been introduced. The reader will, I think, appreciate the lessening of the labour required to find what he may wish to refer to.

W. M. B.

1911.

PREFACE TO FIRST EDITION.

THIS short monograph is based upon lectures given at various times in University College, London, and this fact may serve as the excuse for what some may regard as too definite a position with respect to certain disputed questions. It is, I venture to think, preferable to take a definite point of view, rather than to leave the study of the subject in a state of chaos. At the same time, one must always be prepared to correct one's views when evidence is brought against them. I have endeavoured not to slur over difficulties, where such really exist, as also to indicate the existence of points of view contrary to my own. It is quite possible, however, that, by inadvertence or ignorance, important facts may have been omitted.

Certain subjects of considerable interest in connection with the theory of the action of enzymes, such as the properties of colloids and the laws of adsorption, which were dealt with in some detail in the lectures, are here only treated in their immediate bearing on the facts under discussion.

No attempt has been made to put forward a complete bibliography in the list of literature at the end. This contains only those writings which have a more or less direct bearing on the subject and which are referred to in the text. In this list I have added, after each reference, the page of the text in which it is made use of, so that the insertion of names of authors in the general index is rendered unnecessary.

The detailed description of the properties of the large number of specific enzymes already known finds no place in this monograph, which confines itself to general properties more or less common to all enzymes.

W. M. B.

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CHAPTER I.

CATALYSIS IN GENERAL.

Bio-chemical Reactions.

ONE of the most striking characteristics of the chemical changes taking place in living organisms is the ease with which bodies of a highly stable nature are split up. Glucose, for example, is oxidised to carbon dioxide and water, egg-white is hydrolysed to amino-acids. Under ordinary laboratory conditions, powerful reagents, such as chromic acid and boiling hydrochloric acid, are necessary to effect these decompositions.[1] This fact, which is, of course, familiar to all workers in bio-chemistry, was, at an early date in the history of the science, especially called attention to by Schönbein [1863, p. 344].

Catalysis.

Phenomena of a similar kind are, however, known to chemists to take place in the laboratory, and such reactions have been known since the time of Berzelius [1837, pp. 19-25] as "catalytic" reactions. Their importance increases every day. Oxygen and hydrogen, for instance, at ordinary temperatures combine so slowly that the production of water cannot be detected, the application of a flame or electric spark being requisite. But the presence of a minute quantity of finely divided platinum is sufficient to cause combination to take place at room temperature. Again, the oxidations effected by hydrogen peroxide proceed in many cases at a very slow rate by themselves, but can be enormously accelerated by traces of iron or manganese, as in the well-known method of Fenton [1894]. Another case of interest in connection with enzyme action is the hydrolysis of cane-sugar by acids (ionic hydrogen).

The most profitable way of studying the problem before us is to consider first of all the essential characters of catalysis, as manifested by reactions where the bodies concerned are of known chemical composition.

For this purpose we may conveniently divide reactions into two classes.

[1] Note A at end of book.

I. There are a large number of reactions which are practically instantaneous, those between ions forming the chief part of this class. When a chloride is added to a solution of silver nitrate, a precipitate of silver chloride falls at once. Or, when a strong acid is neutralised by a strong base, the union takes place at once, as we know by the regular titration methods.

II. On the other hand, there are reactions, like the saponification of esters by caustic alkali, which take a measurable time to arrive at their final state.

Now, a "catalyst" is a body which alters the rate of reactions of this latter class (see Ostwald [1903, **2**, i., p. 515]). The change may be either in the direction of acceleration or of retardation, and the reaction may be one that, by itself, either proceeds rapidly or so slowly that it requires special proof to show that it is taking place at all. It is especially to the acceleration of this latter kind of reaction that the name of catalysis is usually given, although, in theory, any change of the rate of any reaction by the addition of a foreign substance comes under the same category.

As examples of catalysed reactions may be mentioned: the inversion (hydrolysis) of cane-sugar by acid (hydrion), the numerous combinations effected by the catalytic agency of platinum in particulate condition, and oxidations by hydrogen peroxide accelerated by ferrous or manganous salts. As a case of slowing of a reaction by a foreign body I may refer to the stopping of the slow oxidation of phosphorus in air by a trace of ether vapour; this kind of action is called "negative catalysis".

Mechanical Schema.

There are certain phenomena which, at first sight, might be confused with those of catalysis, but which must be carefully distinguished from them. A mechanical model will serve to make this clear. If a brass weight of, say, 500 grammes be placed at the top of an inclined plane of polished plate-glass, it will be possible to find a slope of the plane such that the weight will slowly slide down. This represents any reaction taking time to complete. If now the bottom of the weight be oiled (oil = catalyst) the rate of its fall will be greatly increased. We see that, in either case, the weight, if placed at the top of the plane, does not remain there, but, sooner or later, reaches the bottom. It may, however, be kept at the top by some kind of catch or trigger arrangement, in which case it will remain there indefinitely until the catch is released. The amount of energy lost by the weight in its fall, being the product of its weight and the vertical height from which it has fallen, is in no way affected by the work required to remove the obstacle

preventing its fall, nor is the rate at which it falls when set free. A typical instance of such a "trigger" action is that of supersaturated solutions, which remain for any length of time unchanged unless infected with a crystal. It has, moreover, been shown by B. Moore [1893] that the rate at which the solidification of supercooled glacial acetic acid moves along a tube is independent of the quantity of crystals placed at one end to start the process. Not so with true catalytic action; although the work done by our sliding weight is in no way affected by the amount of catalyst (oil) used, the rate of the fall is, within limits, directly proportional to it, and this is a property of catalysts in general.

It cannot be expected that a rough model of this kind would show all of the characteristics of catalytic phenomena, but there are two instructive points shown by it in addition to those already spoken of. The first is the disappearance of the catalyst by sticking to the glass as the weight slides down. An analogous phenomenon is often met with in catalytic processes, as will be seen later. The second point is one of importance with regard to certain enzyme actions; it consists in the fact that, although the presence of the catalyst neither adds to nor subtracts from the total energy change in the reaction, the form of this energy may be altered. When the weight falls slowly by itself, nearly the whole of the energy appears as heat due to friction along the glass plane, so that the weight arrives at the bottom with very little kinetic energy; on the contrary, when oiled, nearly the whole of the energy is present in the weight at the end of its fall as kinetic energy, very little friction having been met with in its descent. We may notice, also, comparing the effects of different amounts of oil, that small amounts produce a much more marked result than the subsequent addition of further quantities. This is also characteristic of enzymes, as we shall see later.

From what has been said it follows that a catalyst is merely capable of changing the rate of a reaction already in progress. In opposition to this it may reasonably be said that a reaction does sometimes seem to be initiated. Such a case is that of a mixture of oxygen and hydrogen gases caused to combine by spongy platinum. Now there are reasons for the belief that an extremely slow combination is taking place at ordinary temperatures without catalysis. One thing to be considered in reference to this belief is the enormous acceleration of chemical reactions by rise of temperature, the majority being about doubled by a rise of 10° C. In this way a reaction having a velocity of 1 at 0° would reach one of 2 at 10°, 4 at 20°, and $1 \times 2^{10} = 1024$ at 100°. At the temperature of 500° there is appreciable formation of water in the case in point, and Bodenstein [1899, pp. 694 and 681] has shown that, if the

velocity at 689° be represented by 163, that at 482° has already sunk to 0·28 ; so that at room temperature the velocity would be quite incapable of detection by chemical means, since centuries would be needed to produce a fraction of a milligramme of water. Grove's gas battery also proves that the two gases are not in equilibrium at ordinary temperatures, since electrical energy is obtained by their slow combination.

To take another case of a reaction which progresses at a slow rate when left to itself: when methyl acetate is mixed with water at ordinary temperatures it is very slowly hydrolysed to alcohol and acetic acid until a certain proportion of it is decomposed, so that a state of equilibrium is finally arrived at. This process takes many days for its completion, but the time may be shortened to a few hours by the addition of a small amount of hydrochloric acid.

The objection may be made to the former of these two examples that the combination of oxygen and hydrogen does not take place except in the presence of water vapour, which probably acts as a catalyst. Similarly, the hydrolysis of esters by water may be said to be due to the hydrion present therein. This point of view does not, however, in reality, affect the reasoning, since the reactions can be enormously accelerated by other bodies, which act as additional catalysts and may be investigated independently. It is, in fact, a matter of considerable difficulty to discover a slow reaction which is definitely known to take place in the complete absence of any catalyst.[1]

Moreover, it must not be forgotten that, as J. J. Thomson and others believe, a catalyst may possibly start a reaction. This is not, theoretically, in disagreement with the view taken by Ostwald. To return to our mechanical illustration, the "friction" between the weight and the glass plane may be sufficiently great to prevent movement altogether, until oil is applied. But the use of the name "friction" implies the idea of movement and the existence of forces tending to produce it. One may indeed suppose that the weight actually does move for an infinitesimal distance, but is at once arrested by the resistance met with. From this point of view the definition of a catalyst would be expressed somewhat thus: A substance which changes the rate of a reaction which is actually in progress, or which is capable of proceeding without any supply of energy from without, if certain resisting influences are removed. The difference between diminution of friction by oil and the removal of a catch is that, in the former case the action is continuous throughout the fall of the weight, whereas in the latter case the action is only momentary, at the commencement of the fall, on the rate of which it has no further effect.

[1] See H. E. Armstrong [1885 and 1886].

Criteria of Catalysts.

Several other properties of catalysts must be referred to. They do not appear chemically combined with the final products of the reaction, which are, as a rule, the same as those of the non-catalysed reaction. They are found at the end unchanged, except in those cases in which they are destroyed by subsidiary reactions, such as the nitric acid in the old chamber process of sulphuric acid manufacture.

Since the addition of a catalyst only serves to hasten a result which would ultimately be arrived at by itself, it follows that the effect of a small quantity of catalyst is, in the end, the same as that of a larger quantity, if sufficient time be allowed. This is often in practice a useful criterion in deciding whether a result produced by the addition of a body is due to a chemical reaction following the laws of combining proportion, or to a catalytic process. In the first case the amount of the new products formed will be in exact ratio to the quantity of the body added, in the second case they will be independent of this factor; the only difference between the reactions when large or small amounts of the foreign body are added, if it acts as a catalytic agent, is the rate at which the new products appear. Of course, this last statement is true only when the catalyst is not paralysed or destroyed before it has completed its work. Cases are known, in fact, where such phenomena happen, so that the final result depends on the amount of catalyst added; such cases are not very infrequent amongst enzyme actions and will be considered later.

Although the degree of acceleration of a reaction is proportional to the concentration of catalyst present, it is astonishing how minute a quantity is capable of perceptible activity. For example, according to Bredig and v. Berneck [1899, p. 276], colloidal platinum is able to act upon 1,000,000 times its weight of hydrogen peroxide; again, it was found by Brode [1901, p. 289] that, in the reaction between hydrogen peroxide and hydriodic acid, the catalytic action of 1 gramme-molecule of molybdic acid in 31,000,000 litres could still be detected.

Catalysis of Reversible Reactions.

The question naturally arises, when the reaction catalysed is a reversible, or balanced, one, has the fact that the equilibrium position is arrived at under the accelerating influence of a catalyst any effect on the actual position of this equilibrium? It was shown by Berthelot and Péan de Saint-Gilles [1862, p. 418] that, if 1 mol. (= gramme-molecule) of ethyl alcohol be mixed with 1 mol. of acetic acid, the reaction proceeds until the mixture has the composition :—

$\frac{1}{3}$ mol. alcohol + $\frac{1}{3}$ mol. acid + $\frac{2}{3}$ mol. ester + $\frac{2}{3}$ mol. water.

The same end-position is also reached when 1 mol. of ethyl acetate is mixed with 1 mol. of water. So that, in any mixture of these four bodies, two opposite reactions are proceeding at unequal rates until a certain relative concentration of the constituents results, at which point the two reactions have an equal velocity.

Now suppose that a catalyst, *e.g.* HCl, is added to such a system, it is plain that, unless both the opposite reactions were equally accelerated, the equilibrium-position would be changed. This can only be done by adding or taking away energy, since a change in the position of equilibrium implies a change in the osmotic pressure of the solution. If, then, our definition of a catalyst be correct, *viz.*, that it neither adds nor subtracts energy, it follows that it must accelerate both the hydrolytic and the synthetic reactions. This has been shown experimentally by Knoblauch [1897, p. 269] for the cases of formation and decomposition of esters by hydrochloric acid. As regards the non-alteration of the position of equilibrium, the proof has been given by Koelichen [1900, pp. 136 and 149], in the case of the reversible polymerisation of acetone by the catalytic action of bases, that the equilibrium-constant is, in dilute solution, independent of the concentration or nature of the catalysing base. Similarly, it has been shown by Turbaba [1901] that the equilibrium between aldehyde and paraldehyde is independent of the nature or amount of the catalyst, whether this be sulphur trioxide, zinc sulphate, hydrochloric, oxalic, or phosphoric acids.

The state of affairs described above does not necessarily hold when the catalyst is altered in any way by the reaction ; such alteration may be physical or chemical. The former case is of special importance as concerning enzymes, as will appear later. It has also been shown by Abel [1907, and 1912] that, when the reaction proceeds with the formation of an intermediate combination between the catalyst and the body which is attacked, the law that catalysts must raise, in quantitatively equal proportional amount, the two opposite factors of a chemical reaction, does not necessarily hold. In other words, if the catalyst is in a different

chemical or physical state at the end from what it was at the beginning, it has given up or received energy, so that a change of position of equilibrium is quite conceivable. In this connection it is important to remember that the reactions in which enzymes play a part are very nearly thermoneutral, or occur without any considerable heat change, with the exception of oxidations and reductions. This fact means that the reactions require comparatively little energy to bring them about. It seems, then, that surface energy, which, as we shall see, is an important property of the colloidal state, may be sufficient, as Herzog [1910, p. 196] suggests, to account for the fact that the equilibrium position in a reaction brought about by a catalyst in true solution, such as the action of acid on esters, is not quite the same as when the colloidal enzyme, lipase, is the catalyst.

A further fact, which follows from the kinetic consideration of the position of equilibrium in reversible reactions, is that, if a reaction of such a kind is accelerated by the presence of a catalyst, any equilibrium position other than that of practically complete change in one direction, means that the catalyst has acted on *both* the hydrolytic and the synthetic reactions. For example, the spontaneous rates of these reactions in the case of ethyl acetate is extraordinarily slow, several weeks being required for the attainment of equilibrium. If acid be added, a few hours are sufficient. Now, remembering that the equilibrium position depends on the relative rates of the two reactions, as does the meeting-point of two people walking towards one another at different speeds, it will be obvious that, if the acid accelerated one reaction only, say that of hydrolysis, making it, as it does, one hundred times as fast as the spontaneous one, but leaving the opposite synthetic one untouched, the equilibrium position must of necessity be at such a point that there will be present about one hundred parts of the products of hydrolysis to one part of the synthetic products. Any proportion of synthetic products greater than this one per cent., or so, means that the synthetic reaction has also been accelerated. It is not necessary, then, that the equilibrium position should be the same as that in the absence of a catalyst to prove the acceleration of both components of the reversible reaction. In other words, there may be conditions present which cause one reaction to be affected more than the other, although both must be accelerated unless there is practically complete change in one or other direction. In testing enzymes for synthetic powers, this result must be duly taken into consideration, especially since enzyme actions take place in a heterogeneous system, where perturbations likely to affect the precise position of the equilibrium point are probable. This fact is apt to be overlooked.

Mode of Action of Catalysts.

It seems probable that the explanation of a large number of catalytic reactions lies in the formation of intermediate compounds. At the same time, as Ostwald (see Brode [1901, p. 305]) has pointed out, in order that this shall be a satisfactory theory it must be shown, in any given case, that the sum of all the reactions when intermediate compounds are formed is more rapid than the uncatalysed reaction itself. Brode [1901, p. 281, etc.] has given the direct proof of one case of such a reaction. Hydriodic acid is decomposed by hydrogen peroxide at a measurable rate, which is enormously increased by molybdic acid acting as a catalyst. During the course of the reaction a series of permolybdic acids can be shown, by chemical means, to be formed and the velocity of their production determined. These acids decompose hydriodic acid with great rapidity, so that the sum of the two reactions, formation of permolybdic acids by action of hydrogen peroxide on molybdic acid and decomposition of hydriodic acid by these, takes place at a considerably greater rate than that at which hydrogen peroxide by itself can effect the change.

The theory of intermediate compounds does not explain all cases. For example, Tafel [1896] has shown that the catalytic action of hydrochloric acid in the formation of esters from methyl alcohol and acids cannot be accounted for by the intermediate production of methyl chloride (CH_3Cl), as had been suggested. In the first place, this body is not formed with any rapidity under the conditions of the reaction, and in the second place, when added, it is found unable to replace hydrochloric acid as a catalyst.

Catalysis in Heterogeneous Systems.

It would be unfruitful, in the present state of knowledge, to discuss in further detail the various hypotheses put forward to explain catalysis. One of these, however, is of importance in connection with colloidal catalysts, such as enzymes are, namely, surface-condensation of the reacting bodies, in which case the accelerated rate of change is, in all probability, due to increase of concentration. A similar hypothesis was suggested by Faraday to explain the action of platinum on mixtures of oxygen and hydrogen gases.[1]

Since enzymes are colloids, reactions catalysed by them take place in heterogeneous systems of, at least, two phases. The factors governing the velocity of such reactions are, in addition to the chemical change itself, certain physical processes, such as diffusion, adsorption, etc. The discussion of the mechanism of these reactions will, therefore, be more profitably undertaken at a later stage, when the properties of colloids have been considered.

[1] See Mellor's *Chemical Statics and Dynamics*, p. 258 *seqq.*

Catalysts are Definite Chemical Compounds.

Attention must be called to one more point before passing on to the consideration of the special class of catalysts known as "enzymes"; in view of certain theories as to the nature of enzymes, it is important to notice that all the catalysts mentioned in this chapter are definite chemical individuals of known composition and properties. As yet this statement cannot be made of any enzyme. We are not, however, warranted in denying definite chemical constitution to this latter class of bodies, until it has been shown that bodies of known constitution may at one time possess the properties of enzymes and at another time, without any change in their chemical nature, be devoid of such properties.

As instances of organic compounds of known composition which have been shown to act as catalysts, Dakin's work with amino-acids [1909] and that of Fajans on nicotine [1910] may be referred to.

For a more detailed account of the general phenomena of catalysis Chapters X. and XI. of Mellor's *Chemical Statics and Dynamics* should be consulted, and also the voluminous work by G. Woker [1910].

CHAPTER II.

ENZYMES AS CATALYSTS.

History.

AT an early date in the history of physiology bodies having properties similar to those of the inorganic catalysts were prepared from the tissues of living organisms. In 1830 Dubrunfaut prepared an extract of malt which converted starch into sugar [1830], just as strong acids were known to do (Kirchhoff [1815]). Three years later, Payen and Persoz precipitated by alcohol from such extracts a substance which could be dried and preserved, and which had a very powerful action on starch [1833]. This they called "diastase".

As more bodies of similar properties became known, they were called "ferments," on account of the resemblance of their activities to those of alcoholic fermentation.

When Pasteur had shown that this process was due to the presence of a living organism, diastase and bodies like it were distinguished as "soluble or unorganised ferments" in contradistinction to living organisms, like yeast, which went by the name of "organised ferments".

Although many physiologists, as Traube for example, were of opinion that organised ferments owed their properties to the presence within them of soluble ferments, the double use of the word "ferment" gave rise to some confusion, and induced Kühne [1878, p. 293] to suggest a new name in an interesting passage, of which the translation is :—

"The latter designations (*i.e.*, formed and unformed ferments) have not gained general acceptance, in that on the one hand it was objected that chemical bodies, like ptyalin, pepsin, etc., could not be called ferments, since the name was already given to yeast-cells and other *organisms* (Brücke); while on the other hand it was said that yeast-cells could not be called *ferment*, because then all organisms, including man, would have to be so designated (Hoppe-Seyler). Without stopping to inquire further why the name excited so much opposition, I have taken the opportunity to suggest a new one, and I give the name *enzymes* to some of the better-known substances, called by many 'unformed ferments'. This is not intended to imply any particular hypothesis, but it

merely states that ἐν ζύμῃ (in yeast) something occurs that exerts this or that activity, which is considered to belong to the class called fermentative. The name is not, however, intended to be limited to the invertin of yeast, but it *is* intended to imply that more complex organisms, from which the enzymes, pepsin, trypsin, etc., can be obtained, are not so fundamentally different from the unicellular organisms as some people would have us believe."

In this monograph the name "enzyme" will therefore be invariably used.

Definition.

We may, then, for the present define enzymes as the catalysts produced by living organisms. This statement is not to be taken as in any way prejudging the possibility of their ultimate production in the laboratory; when a body of the properties of trypsin is synthesised it will have every right to the same name. In the actual investigation of enzymes their source is, of course, immaterial.

It is held by E. F. Armstrong [1913] that the definition given above should be qualified thus—enzymes are "selective, colloidal catalysts, present in living cells and destroyed by heat". I am unable to agree to this limitation, although by not doing so the definition becomes less definite. We must remember, however, that, in point of fact, it is merely a matter of practical convenience to separate enzymes from other catalysts and that the separation, from a theoretical standpoint, has its disadvantages. When the actual chemical constitution of enzymes is known they will not cease to be enzymes, although their source may not be from living cells exclusively. Perhaps, in the future, the name "enzyme" or "ferment" may have to be given up, as applying to a distinct class of substances. Moreover, to apply the name "selective" to such an enzyme as emulsin, which acts on so great a variety of compounds, seems rather out of place, and there are also simple inorganic catalysts which may quite properly be called "selective," such, for example, as the reaction between hydrogen peroxide and a thiosulphate, as catalysed, on the one hand, by iodine ions, and, on the other hand, by molybdic acid, described by Abel [1912]. Rhodium, according to Deville and Debray [1874], in a certain state, acting as catalyst on formic acid, produces carbon dioxide and hydrogen, while platinum cannot be brought into this state. Falk [1913] gives good reason for regarding the apparent specificity of lipase as due to the chemical action of the various substrates on the enzyme itself. For further discussion of this part of the question, see Note D in the Appendix.

The introduction of the word "colloidal" is also, as I think, inappropriate. A typical inorganic catalyst, such as the hydrosol of platinum, is in the colloidal state, as are enzymes as a general rule. But, in some instances, it appears to be doubtful whether the really active constituent of the enzyme system is itself in this state. It may be a constituent of an adsorption compound with a colloid. As to destruction by heat, it is undoubtedly, in many cases, a useful criterion, but the actual temperature required varies so much according to conditions that it does not seem advisable to make it a necessary property of an enzyme. Some inorganic catalysts, also, are sensitive to heat.

In the above definition, as will be noted, it is assumed that enzymes behave as catalysts, so that it is incumbent upon us at the outset to consider how far the statement is justified. As is pointed out by A. E. Taylor [1910-11], much of the difficulty experienced by some investigators in regarding enzymes as catalysts is due to incorrect conceptions of the meaning of catalysis.

Berzelius [1837], in his *Lehrbuch der Chemie*, had already called attention to the similarity of enzymes and catalysts in the following words: "We had the experience of finding that the change of sugar into carbon dioxide and alcohol, as it takes place in fermentation under the influence of an insoluble body, which we know by the name of ferment, could not be explained by an action similar to double decomposition between the sugar and ferment. But, when compared with known phenomena in the inorganic world, it resembled nothing else so much as the decomposition of hydrogen peroxide under the influence of platinum, silver, or fibrin; it was therefore quite natural to assume an analogous action in the case of the ferment." And again: "We have reasons, well founded on fact, to make the assertion, that in living plants and animals there take place thousands of catalytic processes between tissues and fluids".

Views of a similar kind have been expressed by physiologists, Carl Ludwig, Traube, Bunge, and many others.

Terminology.

Before proceeding further with the question before us, it is advisable to refer briefly to the terminology of enzyme action in general. A name is frequently needed for the substances on which enzymes exert their activity. Unfortunately, no really satisfactory name has yet been suggested. "Hydrolyte" would serve where the action is one of hydrolysis, but it would exclude any other action such as oxidation or intramolecular splitting, as well as the synthetic actions, such as the formation of a disaccharide by the action of maltase on glucose. On the whole, "substrate," already used by many writers, seems to answer the purpose best. The objection to the name "zymolyte" proposed by Loevenhart and Peirce [1907] is that it applies only to the case of enzymes and excludes other catalysts. The suggestion of difference is, I think, to be deprecated.

As to the names of the enzymes themselves, it was suggested by Duclaux that the termination "ase" should be taken as denoting an enzyme, and that this termination should be added to the name of the substrate, *e.g.*, lactase is the enzyme accelerating the hydrolysis of lactose. It would be inconvenient to displace old-established names, such as "pepsin" and "trypsin," but, as far as possible, the recommendation of Duclaux should be acted upon.

It has been the custom to speak of an enzyme which attacks, say, starch or protein, as "amylolytic" or "proteolytic" respectively; but, as Professor Armstrong has pointed out, these names are incorrectly formed. "Amylolytic," in analogy with "electrolytic," should mean a decomposition by means of starch. To avoid this misuse of words, Professor Armstrong [1890] advocates the use of the termination "clastic" instead of "lytic" in speaking of the action of an enzyme on its appropriate substrate.

An unfortunately chosen termination "ese," as expressing a synthesising enzyme, has recently been introduced by Euler [1911]. But there is no evidence that such enzymes exist, as distinct from those producing hydrolysis, as we shall see later, so that the termination is unnecessary.

Catalytic Properties of Enzymes.

Apart from theory, it is useful to know what kind of properties to look for in a substance suspected to be an enzyme. An unknown body, if an enzyme, may be expected to manifest the general characters of catalysts to a greater or less degree.

Now we have seen that there are practically only two properties common to all catalysts, *viz.*, that of not initiating a new reaction, but merely changing the rate of one already in progress, and that of not appearing in the final products of the reaction, which is accelerated. Of these the latter is the more important. In a catalytic reaction there is no proportionality between the amount of the catalyst present and that of the final products. The catalyst either reappears at the end unchanged, which is the usual and typical case, or, in some instances, it may be changed or destroyed by a subsidiary reaction which has no connection with the main reaction. Certain other properties are consequences of one of these two, while others, although possessed by the majority of catalysts, do not seem to be essential. We may now proceed to examine, in order, these various properties as manifested by enzymes.

It is obviously not an easy matter to give a satisfactory answer to the question whether enzymes start a new reaction or accelerate one already in progress. The state of affairs is, in fact, similar to that of a mixture of oxygen and hydrogen gases catalysed by platinum, in which we found evidence that the combination does take place at room temperatures, although at an inappreciable rate. The greater number of enzymes have a hydrolytic action, and their activities are, as a rule, manifested in the presence of an excess of water. Now we know that water contains hydrogen- and hydroxyl-ions, which have a hydrolytic action, and although their concentration is very small at 0°, it increases rapidly as the temperature rises. The following numbers will serve to show this :—

Gramme-ions of $H^{\cdot}$ per litre of water at :

0° . . .	$0{\cdot}35 \times 10^{-7}$
18° . . .	$0{\cdot}80 \times 10^{-7}$
50° . . .	$2{\cdot}48 \times 10^{-7}$ (Kohlrausch).

In fact, water at 100° is capable of hydrolysing cane-sugar to glucose and fructose at a measurable rate. Munk [1877-78, p. 395] found that, at 150°, salicin undergoes hydrolysis in water. It is, therefore, no unjustifiable assumption that the process takes place at room temperature, although slowly. Changes at this temperature have, moreover, been described. Starch solutions were found by Aggazzotti [1907]

to undergo a spontaneous change in the direction of dextrin and sugar. Brailsford Robertson [1907, **1**, p. 344] also noticed that solutions of ammonium caseinogenate slowly increased in electrical conductivity when left to themselves, a change similar to that which occurs when they are acted upon by trypsin.

As we have seen in the previous chapter, it does not really affect the theory of catalysis whether the reactions accelerated by catalysts are already in progress owing to the action of another catalyst, such as, it may reasonably be held, $H^{\cdot}$ or OH' ions, in the examples just given.

Variety of Products.

Certain difficulties must not be overlooked. Enzymes, in many cases, do not carry the hydrolytic process as far as acids do. The amylase of malt converts starch into maltose and apparently no further, whereas acids convert it into glucose; trypsin acts on proteins leaving unattacked a complex polypeptide, which can be further split into amino-acids by acids or by the enzyme, erepsin. Other cases might be mentioned, but these will suffice to illustrate the point. With regard to this objection, there are one or two considerations to be borne in mind which tend to remove its serious nature in some degree. In the first place, it is not impossible that all these reactions would continue to complete hydrolysis, if appropriate conditions were present; we shall see later that an enzyme action comes to an end- or equilibrium-point owing to the accumulation of the products of the reaction, so that by dilution, or removal of the products, the reaction can be caused to go on farther. The final part of the change may be very slow, so that very prolonged periods of action may be necessary to detect it; the case of pepsin is instructive in this connection; it was thought until recently that this enzyme was unable to hydrolyse proteins beyond the stage of "peptones"; we know now that, if sufficient time be allowed, amino-acids are produced.[1] Also malt extract, if allowed to act on starch for a long time, produces glucose. The formation of alcohol from sugar is another difficult case. It appears moreover that different enzymes produce different bodies from the same substrate. The weight falling along an inclined plane, as used for an illustration in the preceding chapter, gives us a hint here; it was pointed out that, although the total energy change was the same whether little or much catalyst (oil) was present, yet the form of energy might be quite different, in the one case heat, in the other kinetic energy.

Notwithstanding what has been said, the fact remains as yet not

[1] See note B.

satisfactorily explained why one enzyme effects part of a change as rapidly as another enzyme, for example, the production of peptones by pepsin and trypsin, while amino-acids are rapidly formed by the latter enzyme and but very slowly by the former.

In this connection the experiments of Duclaux [1896] are very suggestive. It was found that, in the presence of sodium hydroxide, glucose was decomposed by light with the production of ethyl alcohol, whereas, if the sodium hydroxide were replaced by calcium hydroxide, lactic acid was formed. The reaction stops before completion when calcium takes the place of sodium. Differences in the physical properties of calcium and sodium lactates may possibly be the cause of this phenomenon. It serves to show that it is not necessary to assume any fundamental difference between acids and enzymes because the former appear to carry the hydrolytic process further than the latter.

An interesting comparison between the action of pepsin and of dilute hydrochloric acid has been carried out by Abderhalden and Steinbeck [1910, 1]. Peptone is attacked by neither. Natural albumin (egg or serum) and gelatin are attacked about equally, but when they have been coagulated by boiling, gastric juice hydrolyses them, while dilute hydrochloric acid does not. There is an obvious connection between the physical state of a substrate and the action of different catalysts.

Were it not that it seems impossible to place enzymes in any other category than that of catalysts, the anomalies above touched upon would be more serious. It is quite evident that chemical reactions in combining proportion are out of the question, since the enzyme is not a constituent of the final products and the amount of these is independent of the amount of enzyme added. Moreover, anything of the nature of "trigger action" is excluded by the fact, familiar to all who have made experiments with enzymes, *viz.*, that the effect as regards the rate of action is directly proportional to the concentration of the enzyme.

On the whole, considering how little we know as yet about the intimate nature of catalytic phenomena in general, there is no doubt that the difficulties referred to will sooner or later be removed.

Final Result Independent of Amount of Catalyst.

The next point to examine is the behaviour of enzymes with respect to the final result. We have seen that in catalysis this is independent of the quantity of catalyst present, provided that the latter has not disappeared from the sphere of action before the end-point is reached. Fig. 1 (p. 18) will serve to show this in the case of trypsin. The series of curves represent the increase in electrical conductivity in a 5 per cent. caseinogen solution under the action of various relative amounts of the same enzyme preparation, as marked on the curves. The changes in conductivity are, as I have shown [1907], proportional to the amount of peptone and amino-acids produced. Several things are to be noticed in these curves. The rate of the change, up to about the eightieth minute, is directly proportional to the concentration of the enzyme, as seen in the relative steepness of the curves. As the reaction goes on, one curve after another joins that of the larger amount, until finally they all arrive at the same position. The one with the least amount of enzyme did not arrive at this height during the time shown in the curve, probably because of the destruction of the trypsin, which is an unstable body. The last fact shows the necessity of care in interpreting the results of experiments made for the purpose of tracing the relation between concentration of enzyme and effect produced. It was, at one time, denied by some observers that Buchner's zymase, the alcohol-producing body of yeast, had the characters of an enzyme. But if the tables on pp. 160-62 of Buchner's *Zymasegärung* [1903], be consulted, it will be seen that its behaviour closely resembles that of trypsin; it is probable that the zymase is destroyed by the proteoclastic enzyme present with it in the yeast-juice. As regards this enzyme, zymase, however, recent work of Buchner and Klatte [1908], shows that, in many instances at least, the loss of activity of kept juice is due to the disappearance of the co-enzyme of Harden and Young (see Chap. VIII. below), so that the activity can be restored by the addition of boiled juice.

Many enzymes, such as amylase, can be found at the end of the reaction apparently unaltered (Effront [1899, p. 155], and Starkenstein [1910]), and can then act upon a further supply of substrate. Even trypsin, when in presence of a high concentration of substrate or products, is not at all rapidly destroyed. This circumstance is sometimes made use of in preparing enzymes from the cells in which they occur, *e.g.*, pepsin from gastric mucous membrane, by allowing the tissue to digest itself in presence of acid at 40° C.

The fact that a small amount of enzyme is as equally effective as a

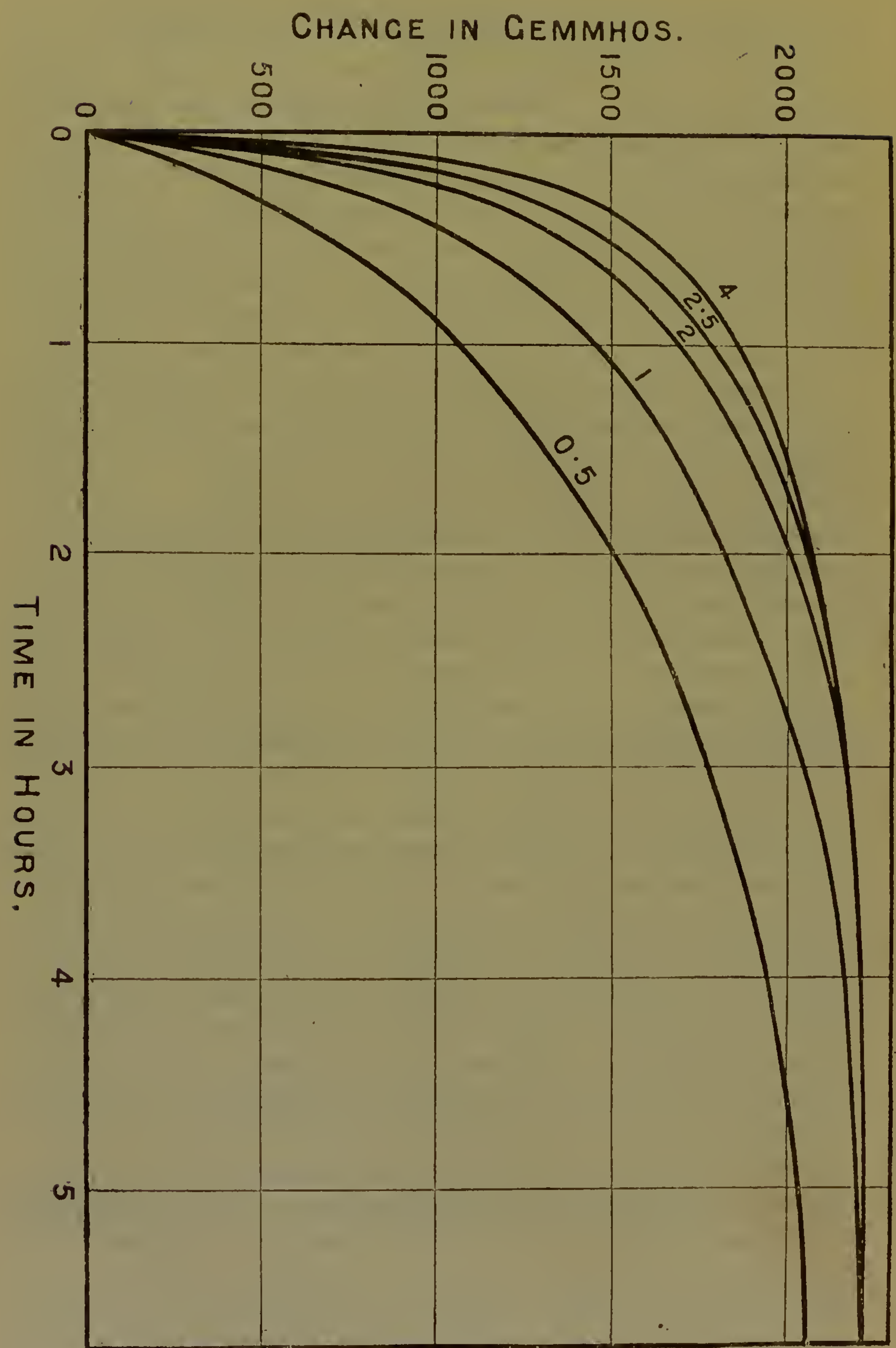

CHANGE IN GEMMHOS.
0
500
1000
1500
2000
TIME IN HOURS.
0
1
2
3
4
5
4
2.5
2
1
0.5

FIG. 1.

larger, if sufficient time be allowed, was made use of by the author in conjunction with Starling to decide the dispute as to the nature of enterokinase [1905]. This body, discovered by Chepovalnikoff[1] in the *Succus entericus*, has the power of converting the inactive zymogen of the pancreatic juice, as secreted, into active trypsin. In the opinion of Pavloff, the body has the nature of an enzyme, whereas Delezenne and others regard the action as consisting in the chemical union of the two bodies to form the third, trypsin. If this latter view be correct, the quantity of trypsin formed will be in exact proportion to the amount of enterokinase used. We found, on the contrary, that quantities of the enterokinase solution, varying from 0·0001 c.c. to 1·6 c.c. per 10 c.c. of juice, if allowed to act for two days, produced the same amount of trypsin, within the limits of experimental error. In using the method for such a purpose it is obviously essential to take care that the least amount added is not large enough to combine with the whole of the body to be changed, otherwise there would, even in case of a true chemical compound, be no difference between the action of large and small amounts. The kind of results obtained in an experiment of the kind mentioned are seen in the table below.

The first column gives the quantity of enterokinase solution in 11·6 c.c. of secretin juice, the other three the length in mm. of gelatin of Mett's tubes digested in the time noted at the head of the column.

Enterokinase added c.c.	In first six hours.	In next eighteen hours.	In next twenty-four hours.
0·1	0	4·1	10·5
0·4	2	10	9·25
1·6	3	11	9

Owing to the absence of appreciable quantities of substrate or products a slight destruction of trypsin had taken place in the cases where it had been formed at an early stage.

[1] Thesis, St. Petersburg, 1899. Referred to by Pavloff in "Le Travail des glandes digestives". Paris, 1901, p. 257.

Minute Quantities Active.

Instances were given in the previous chapter of the minute traces in which catalysts are able to exercise their action. This is, of course, not surprising when the catalyst remains unchanged at the end of its work; at the same time it is perhaps striking that such a very small concentration should have any effect whatever, no matter how long it were allowed to act. In the case of enzymes we find similar facts. Invertase, according to O'Sullivan and Tompson [1890], can hydrolyse 200,000 times its weight of saccharose; rennet, according to Hammarsten, can clot 400,000 times its weight of caseinogen in milk, and so on. When we remember that these preparations consist, in all probability, only to a small extent of the actual enzyme, their activity becomes all the more astonishing.

Effect on Reversible Reactions.

Since, as we shall see in more detail in a later chapter, there are good reasons for the statement that reactions catalysed by enzymes are, as a general rule, reversible ones, it is of considerable importance to know whether those enzymes which are known to accelerate a decomposition process also accelerate the opposite or synthetic process, just as we have seen in the case of inorganic catalysts such as hydrogen ions. The question will come up for discussion in a subsequent chapter, so that I will merely state here that it has been shown for many enzymes that such is the case. Maltase forms glucose from maltose and a disaccharide from glucose, emulsin hydrolyses glycerol-glucoside and also, under different conditions, synthesises it from glucose and glycerol, lipase accelerates the hydrolysis of ethyl butyrate and also its formation from butyric acid and ethyl alcohol, and so on.[1]

As to an influence on the position of equilibrium, we have seen that, if the catalyst acts by the formation of intermediate combinations, this position is not necessarily the same as that of the uncatalysed reaction, or even that attained under the influence of a different catalyst. Now, since enzymes act by forming intermediate combinations, of what kind will be discussed later, it is not surprising to find that the equilibrium position under lipase is not the same as that under hydrogen ion (Dietz, [1907, p. 320]).

In my investigation of the case of emulsin and glycerol-glucoside, I found [1913, pp. 245, 246] that the concentration of the enzyme had no effect on the position of equilibrium, a fact which indicates that the enzyme itself does not form a part of the final chemical system,

[1] Note C.

although it leaves unsolved the difficulty as to the difference between the position of equilibrium when colloidal catalysts, such as enzymes, are used and the position under the action of acid. The question will come up for further discussion later.

Destruction by Heat.

Unlike most inorganic catalysts, enzymes are destroyed by temperatures from 60° to 100°, differing according to the conditions present.[1] This behaviour, though of practical importance, does not affect their claim to be regarded as catalysts and will be explained in the next chapter. The phenomenon of an apparent *optimum* temperature, in which enzymes differ from the majority of other catalysts, will also be explained later and shown to be due to injury or partial destruction by the action of heat.

[1] See, however, p. 97.

CHAPTER III.

PHYSICAL AND CHEMICAL PROPERTIES OF ENZYMES.

Enzymes are Colloids.

ONE of the most important physical properties of enzymes is their colloidal nature, as shown by the fact that they do not pass through parchment-paper, or do so with extreme slowness. This property is, indeed, the only essential one which distinguishes enzymes from other organic catalysts, such as amino-acid in Dakin's experiments, or organic bases in those of Fajans, to which reference was made at the end of Chapter I. Since many other characteristics are dependent on this fact, it is advisable to devote a little space to the discussion of the essential properties of colloidal solutions in so far as they are of importance with respect to the nature and mode of action of enzymes.

Nature of the Colloidal State.

The first thing that strikes an observer as regards colloidal solutions is that bodies which in the usual sense of the word are insoluble in water, metallic platinum or arsenious sulphide, for example, can, by appropriate methods, be induced to "dissolve" in water so as to form solutions which are permanent but in many ways behave differently from true solutions. By various means it can be shown that the body so taken up in water is, in reality, in the state of extremely minute, "ultra-microscopic" particles, that is, particles too small to be visible under ordinary methods of illumination of objects under the microscope.[1] The chief proof that we have to do with matter in a particulate condition consists in the application of the Faraday-Tyndall test. This consists in passing a powerful beam of light through the liquid; if particles be present, the path of the beam can be readily seen as a bright streak, when looked at from the side. In the case of permanent colloidal solutions, which do not deposit on standing, the light sent out at right angles to the beam is found to be polarised; this means that the particles reflecting it are smaller than the mean wave-length of the light forming the beam.

[1] It is pointed out by Hardy [1899, **2**, p. 110] that, according to the phase-rule, these colloidal solutions probably consist of suspensions of solid particles, containing a small amount of water, in a dilute true solution of the solid body in water. These are the two phases of the heterogeneous system. The same view is taken by Quincke [1902, p. 112].

The Ultra-microscope.

If the horizontal beam of the Faraday-Tyndall test be made, by optical means, very narrow and intensely bright and caused to traverse a solution contained in a cell on the stage of a microscope, it can be examined from above by the usual objectives and oculars. Using fairly high magnification, say 4 mm. obj. and ocular 4, the separate particles in many colloidal solutions can be seen as shining diffraction discs, in vigorous Brownian movement. An apparatus of this kind is known as the ultra-microscope of Siedentopf and Zsigmondy [1903].

Properties due to Surface Development.

If now we consider in what respect the particles, say, in a colloidal solution of platinum prepared by Bredig's method of an arc between platinum electrodes under water [1899, p. 265] differ from an equal amount of the metal in the form of sheet or wire immersed in water, it is plain that it consists in the relatively enormous development of surface in the former case. According to Siedentopf and Zsigmondy [1906] the particles in certain colloidal solutions of gold had a radius of one-millionth of a centimetre; a sphere of gold with a radius of 1 millimetre if divided into particles of these dimensions would have a surface of some 100 square metres.

It might, perhaps, be supposed that if the process of subdivision were carried farther and farther until molecular dimensions were reached, the phenomena due to surface would be more and more manifest. In point of fact, however, this is not the case; although we know nothing of what the molecular state essentially is, we do know that bodies in this condition do not show the properties of matter in mass, *i.e.*, bounded by surfaces. At the same time it must be understood that there is no hard and fast line to be drawn between matter in pieces visible to the naked eye, down through ultra-microscopic particles to molecules. Such properties as osmotic pressure, diffusibility, etc., are exhibited by all and in proportion to the dimensions of their elements. Certain of these properties are more pronounced in some of the above stages, others in other stages. For example, osmotic pressure, which is a function of the number of bodies acting as individuals, whether molecules, ions, or particles, in unit volume, will naturally be greater, the greater the number of bits a given mass is divided into; whereas the phenomena due to surface can only be present as long as the particles still have the properties of matter in mass.

There is one respect in which a real distinction seems to exist. An atom or molecule, when it has an electric charge, is called an ion; col-

loidal particles are also capable of carrying a charge, but whereas in the former case this charge is invariable in amount and always the same in sign for each kind of ion, in the latter case the charge is variable, both in sign and quantity. The probable reason for this we shall see presently.

The great distinguishing characteristic of colloids, accordingly, is the enormous development of surface. We must, therefore, look for properties dependent on this. Now the most salient of these is that of surface-tension. When solids are immersed in fluids the film at the interface where the two are in contact behaves as if stretched. Any free surface of a liquid shows this property. Its real existence can easily be made manifest by taking a wire ring with a thread tied to two points so as to lie loosely across the space; the ring is then dipped into soap solution so as to make a film in which the thread floats; if the film be now broken on one side of the thread by touching it with a pointed bit of filter-paper, the thread is immediately drawn into the arc of a circle by the tension of the rest of the film. Suppose, then, that the colloidal particles are suspended in a liquid containing in solution a substance which lowers the surface-tension of the liquid, as in fact the majority of bodies do, it is plain that, if this substance accumulates at the interface of the solid and liquid, the surface-tension at this place will be lowered. It can be shown thermodynamically that such a process tending to a diminution of tension will of necessity occur (Willard Gibbs [1878]). It is, in fact, the phenomenon known as "adsorption," which plays so large a part in the behaviour of colloids, and everywhere where surfaces are in contact with liquids or gases. The phenomenon can readily be seen by taking a dilute solution of a dye such as congo-red and immersing a piece of filter-paper in it; after a time the dye will be seen to have concentrated on the paper, and, if the solution was not too strong, the colour will have nearly vanished from the solution and be transferred to the paper. Such a process is obviously of much importance in cell-activity, where the constituents of the protoplasm are colloids and the various bodies which arrive to be acted upon, or themselves to act, are in very dilute solution. It also plays a prominent part in enzyme action, as will often be seen in subsequent pages.

It is important to remember that a variety of processes which affect the surface energy occur at boundary surfaces between two phases. Such phenomena are electrical charges, changes of solubility, of compressibility, and so on. These must all be taken into account in the discussion of the factors controlling adsorption.[1] Whenever a process diminishes surface energy it will of necessity take place.

[1] See Wo. Ostwald [1909, p. 428 *et seq.*].

Diffusibility.

There are some properties of colloids which need a little more detailed discussion in respect to their connection with the problem before us.

In the first place there is diffusibility. The fact that, as definitely shown by Starling [1895, p. 323, and 1899, p. 318] and by Moore and Roaf [1906], colloids have a measurable, though small, osmotic pressure proves that their particles possess that amount of motion which is necessary to cause an osmotic pressure. They are, therefore, not absolutely indiffusible. Hæmoglobin is, accordingly, found to diffuse slowly into a jelly of gelatin. As to their ability to pass through membranes, this is a question entirely of the structure of the membrane, especially as to the dimensions of its pores. Congo-red, which shows all the usual characters of colloids, will pass slowly through some samples of parchment-paper, but not at all through others. The aniline dyes manifest all possible degrees of diffusibility, in accordance with the dimensions of their ultra-microscopic particles. Similarly, although most enzymes do not diffuse through parchment-paper, it appears that invertase does so to a slight degree, and "diastase," as prepared by Fraenkel and Hamburg [1906, p. 396] from malt, although showing an illuminated cone in the ultra-microscope and therefore containing particles, which were too small to be seen separately, was found to be divided by dialysis into two distinct enzymes, one of which, *viz.*, that which caused the formation of sugar, passed through the paper, while the other, which liquefied starch, was left behind. Wohl and Glimm [1910], however, give reasons for regarding this assumption as unnecessary. It is clear that the substance which passes through parchment-paper is in a state of much finer subdivision, is more highly dispersed, in the language of colloid chemistry, than that which is unable to pass through. It is not surprising that the greater relative surface of the diffusible part should make it more active, since, as we shall see in more detail later, the action is essentially at the surface. The liquefaction of starch is, of course, a preliminary stage in the formation of sugar.

Hysteresis.

In the second place, colloidal solutions exhibit the phenomena known as "hysteresis". While a sodium chloride solution is always the same whatever its previous history, frozen, boiled, or kept for any length of time, colloidal solutions are not the same after experiencing any of these actions. In other words, they are unstable and liable to spontaneous change. Thus, enzymes in solution lose their activity more or less rapidly. Many, like trypsin, slowly lose activity even in the air-dry state, and can only be kept for any length of time in a desiccator in a cool and dark place.

Shaking renders "rennet" inactive, as shown by Schmidt-Nielsen [1909]. This is due, probably, to the phenomena of surface-coagulation described by Ramsden [1904].

Electric Charge of Colloids.

In the third place very many colloids, both organic and inorganic, possess naturally an electrical charge, positive or negative, and those which have not such a charge can be caused to take up one by the action of electrolytes. Arsenious sulphide, as prepared by passing hydrogen sulphide through a solution of arsenious acid, has a negative charge, while ferric hydroxide, prepared by the dialysis of a ferric chloride solution, has a positive charge; congo-red has a negative charge, night-blue a positive one; serum-globulin, in neutral solution, has no charge, in acid solution a positive one, in alkaline solutions a negative one. Coagulated egg-albumin was shown by Hardy [1899, 1] and natural serum-albumin by Pauli [1906] to be without charge in approximately neutral solution. More recent investigations by Pauli and Handovsky [1909] and by Michaelis [1909, 3] have shown that, in truly neutral solution, serum-albumin is electro-negative, being uncharged at a very faintly acid reaction, *i.e.*, hydrion concentration of 10^{-6} or less than one part of acetic acid in 10,000 of water. This value gives the ratio of the acidic to the basic dissociation constants, as pointed out by Michaelis.

The cause of the charge appears to differ according to the nature of the charged body itself. When this consists of particles which dissociate in solution, the charge is, no doubt, connected with this fact. Aluminium hydroxide, giving off negatively charged hydroxidion, is left with an excess of positive charge. Amphoteric bodies, like proteins, may be either positively or negatively charged, as we have seen. In the case of the various inert powders investigated by Perrin [1904, 1905], kaolin, sulphur, silver chloride, etc., it was found that either a positive or negative

charge could be conferred by making the reaction of the water in which they were suspended acid or alkaline. Here it would seem that hydrion or hydroxidion was adsorbed by the powder, which so obtained a charge. Most insoluble powders or solid bodies immersed in water have naturally a negative charge, as shown by Quincke, but the sign of this charge can be reversed by acid (Perrin). The existence of a charge can be detected by exposing the solution to an electric field between electrodes in a tube, and using perferably Whetham's boundary method [1893, pp. 342-45]; the colloid particles will travel towards the pole of opposite sign to that of their own charge (see the investigations of Hardy [1899, **1**, 1905-6, and 1907]).

Electric Charge of Enzymes.

Statements have been made to the effect that enzymes are electrically charged. Probable as this is, it is perhaps premature to lay much stress on the fact until we are sure that we have pure enzymes in our hands. Michaelis [1909, 3] finds malt-diastase positive or negative according to reaction. In neutral solution it wanders to both poles, but chiefly to the cathode. Pepsin, in neutral solution, is negative, but can be made positive by acid. Trypsin is negative in neutral and alkaline solution, positive in acid. According to Iscovesco [1910], pepsin is positive, even when dialysed; the same applies also to catalase. Loeb [1909] states that pepsin and trypsin are electrolytically dissociated, the former as a weak base, the latter as a weak acid. Iscovesco, however, points out that the experimental facts are not completely explained by this hypothesis.

According to Michaelis and Davidsohn [1910, **1**, p. 4], the isoelectric constant (I) of pepsin is about $5 \cdot 5 \times 10^{-3}$; in other words, this is the region of hydrogen ion concentration in which pepsin goes to both poles when a current is sent through its solution. If expressed in terms of its own acidity (R being its dissociation constant), we have

$$R = 5 \times 10^{9}$$

$$\text{since } I = \frac{R^2}{K_w}$$

where K_w is the dissociation constant of water.

Adsorption.

As we have seen, colloids take up, by adsorption, various other bodies, especially if these are also themselves colloids. Although this process occurs when either or both of the bodies concerned is uncharged, it is naturally greatly increased or diminished if they have opposite or similar charges respectively. These "adsorption-compounds," or "colloidal-complexes," as they are called by some when both constituents are colloids, play a very important part in enzyme action, and in fact in physiological chemistry generally. To understand their nature, or rather their chief properties, let us take a concrete case. When electro-negative arsenious sulphide is mixed with electro-positive ferric hydroxide in such proportions that the resulting mixture is electrically neutral, both bodies are precipitated in the form of a complex colloid, and the fluid becomes clear and colourless. It is sometimes stated that this precipitate is soluble in excess of either colloid. If we make mixtures of these in series, beginning with excess of the one and ending with excess of the other, we shall see that the statement does not correctly express the state of affairs. Suppose that we have so arranged the series that the mixture in electrical neutrality and total precipitation is in the middle, we notice that in all the various mixtures there is a precipitate, but that it progressively diminishes as we proceed towards either end, *i.e.*, as the excess of one or of the other colloid becomes greater. Further, since the two colloids used are conveniently of different colours, it is easy to see by their colour that all the precipitates contain *both* colloids, but in varying relative proportion, according to the excess of either colloid in the mixture. The same statement is to be made with respect to the supernatant fluids, all of which contain both colloids. A series of new colloids has therefore made its appearance, consisting of adsorption-compounds of the original colloids in all possible proportions. These compounds differ from those of pure chemistry in that, instead of being in constant combining proportion, they are compounds in varying combining proportion,[1] the proportion being determined by the relative concentration of the two colloids in the mixture.

An important characteristic of adsorption phenomena is the form of the law which governs the composition of the compounds. To understand this expression it is best to take a concrete case again. Picric acid is soluble in water and in ether, but it is more soluble in ether than in water; with constant quantities of water, ether, and picric acid a definite fraction of the latter will be present in the ether, say four-fifths; let us now double the amount of picric acid, the proportion of solvents

[1] See Ostwald [1903, i., p. 1097].

remaining the same as before; as is familiar to all, the amount of picric acid both in the ether and in the water will be doubled. This is an instance of a physical partition in proportion to relative solubility. Take now a case of a purely chemical reaction such as silver nitrate and hydrochloric acid; if to a solution of silver nitrate sufficient hydrochloric acid to combine with the silver be added, the whole of the latter will be precipitated, so that the addition of more hydrochloric acid will bring down no further quantity. Take finally a case of an adsorption reaction between colloids, cellulose in the form of filter-paper, which acts precisely like a colloid, and a dilute solution of congo-red. After a certain time it will be found that part of the dye has deposited itself on the paper, but not the whole; that is, the reaction partakes of the nature of both the physical and chemical reactions; physical, in that there is dye both in the paper and in the solution, chemical, in that, like the silver chloride, there is a kind of precipitation. If we double the concentration of the dye we shall find that, unlike the chemical reaction, more congo-red is deposited on the paper; and unlike the physical process, it will be found, on making quantitative experiments, that the amount of dye taken up is not doubled, but something less, in fact multiplied by some root of 2; in most adsorption processes this root is found to be less than the square root. Expressed in algebraic form, when the concentration of the solution is doubled, the amount adsorbed is not multiplied by 2, but by 2 to the power $\frac{1}{n}$ where n is greater than 1, and usually less than 2, *viz.*, from 1·4 to 1·7. It is obvious, however, that we are justified in applying the name of adsorption to cases of any values of n, less than infinity,[1] in which latter case the reaction is purely chemical; $\frac{1}{n}$ here becomes 0, and all numbers to the power 0 become unity; in other words, the amount of silver chloride in the example above is always the same whatever the concentration of the hydrochloric acid. When $n = 1$ it is plain that the physical process of partition according to solubility is expressed.

We see then that the important point about the process of adsorption is that relatively more of the compound is formed in the more dilute solutions. Many important facts in the chemistry of colloids, especially in the relation of toxin to antitoxin, find their explanation in this circumstance.

It is of interest to consider for a moment what the expression above

[1] Freundlich [1906], who has done much important work on this subject, is frequently understood as limiting the title of "adsorption" to reactions with particular values of this exponent. It does not appear from his work that he intended to do this.

given means when n is less than 1 ; it must refer to cases where relatively *more* of the adsorption-compound is formed in the stronger solutions. Although such cases are rare, it appears that they may occur.

It will readily be understood that, although adsorption can take place between uncharged colloids, or even those of similar charge, the process is much facilitated when the bodies have charges of opposite sign. The fact is well shown by the relation of paper to colloidal dyes. In water, paper has a negative charge,[1] congo-red also is electro-negative, accordingly there is practically no adsorption. Night-blue, on the other hand, is electro-positive, so that it is adsorbed in large amount (Bayliss [1906, **1**, p. 205]).

Permanency of Suspensions.

Before proceeding to the subject of the influence of electrolytes on colloids, we may refer briefly to the causes of the permanency of colloidal suspensions. There are several causes which have been suggested, and, no doubt, in many cases these co-operate. We have seen how neutralisation of electric charges results in precipitation in some cases, hence it was suggested by Hardy [1899, **2**, p. 114] that mutual repulsion of similarly charged particles was in some cases responsible for the permanency of the solution. But there are many uncharged colloids equally permanent, so that there must be some other reason. Probably the very minuteness of the particles prevents their deposition, owing to the relatively great frictional resistance ; the fact that these particles are in continual motion, like those of gases, must also be remembered. This is shown by their osmotic pressure, as also by the fact that they can diffuse in opposition to gravity, as can be seen by hæmoglobin passing into a tube of solidified gelatin suspended mouth downwards in a solution of this body.

[1] See Winkelmann, *Handbuch der Physik*, 2te Auflage, 1905, p. 955.

Osmotic Pressure.

According to Einstein's theory of Brownian movement, the truth of which has recently been shown experimentally by Perrin [1908-1910] in an almost complete manner, the kinetic energy of a particle is identical with that of a molecule of the fluid in which it is suspended and therefore with that of any other molecule. As far as osmotic pressure is concerned, therefore, particles, molecules, and ions are of equivalent effect, acting in proportion to their number in unit volume.

When colloids are electrolytically dissociated, as are solutions of many dye-stuffs and salts of proteins, the ions must be responsible for a large part of the very considerable osmotic pressure shown by such colloids (Roaf [1910], Bayliss [1909]). Since the organic ion arising from the electrolytic dissociation of these salts cannot, owing to its dimensions, pass through the parchment-paper membrane of the osmometer used, the inorganic ion is also constrained to remain inside, owing to electrostatic forces between the oppositely charged ions. If either ion were able to escape without the other, there would be established a difference of potential between the two sides of the membrane and the possibility of a perpetual motion.

The relative part played by colloidal aggregates, molecules, and ions is governed by a complex equilibrium, which requires further investigation.

Action of Electrolytes.

We have seen that an electrically charged colloid is able to throw down a colloid which has a charge of the opposite sign ; it is therefore not surprising to find that electrolytes have the same kind of effect. Arsenious sulphide, being negative, is precipitated by positive ions. Ferric hydroxide, being positive, is precipitated by negative ions. Now of course in adding an electrolyte, sodium chloride, for example, we are adding both positive and negative ions, which will have opposite effects. In point of fact, in these comparatively simple cases the ion which precipitates is found to be prepotent. How do we know that it is the one of opposite sign? The answer is by comparing the effects of series of salts with common kation or anion respectively. The chloride, sulphate, and phosphate of sodium are nearly equal in action on negative colloids, whereas on positive colloids the effect of the sulphate is considerably more than double that of the chloride, and that of the phosphate very much more than three times.

Organic colloids, *e.g.*, serum-globulin, can be made either positive or negative by acid or alkali respectively, as previously stated. When positive they are precipitated by negative colloids or ions, such as arsenious sulphide or the ferrocyanic ion. When negative, positive colloids or ions are the active agents.

Complex Colloidal Systems.

The possibilities when mixtures of various colloids and ions occur, as in the living cell, will thus be seen to be extremely complex, indeed as yet but little is definitely known of them. The case of two colloids and an electrolyte is comparatively simple and may be mentioned. Paper and congo-red, which are both negative, have but little adsorptive affinity for each other; the presence of positive ions, however, causes a large adsorption to take place, while anions have very little effect. Unlike what happens in this comparatively simple case, if we take negative paper and positive dye, the action of an electrolyte is also that of the kation; so that *less* adsorption takes place than in the absence of the electrolyte [Bayliss, 1906, **1**, p. 203]. The explanation of both cases is, no doubt, that the kation having a charge of opposite sign to that of the paper, causes a diminution or reversal of this latter. The adsorption of a negative dye will therefore be facilitated while that of a positive dye will be obstructed.

Physical and Chemical Aspects.

It is very important to bear in mind that all these colloidal reactions have both a physical and chemical aspect. Certain colloids have a special adsorptive affinity for one another, which is not purely chemical, since it follows the laws of adsorption and not those of constant combining proportion. This fact is of great importance with regard to enzymes.

To avoid misconception, attention must be called to the fact that, as will probably have been noticed, many reactions can equally well be described in the language of pure chemistry or in that of colloidal or physical chemistry. These are in no sense antagonistic, but call attention to different aspects of the phenomena. For instance, the well-known test for proteins with potassium ferrocyanide and acetic acid may be stated to be due to the insolubility in acid of the compound of the protein with the ferrocyanide; or it may be explained by saying that the protein in neutral solution, being a colloid without electrical charge and hence unaffected by small amounts of electrolyte, becomes capable of precipitation by the quadrivalent anion of potassium ferrocyanide when itself made electro-positive by the action of hydrion.

For further information on the important subject of colloids the reader is referred to the forthcoming monograph in this series by Mr. Hardy.

Purification of Enzymes.

Enzymes then are colloids and have the property of carrying down with them, by adsorption, constituents of the solutions from which they are precipitated. It is not therefore to be wondered at that amylase or invertase, as obtained in the usual way, gives carbohydrate reactions, and that pepsin or trypsin gives protein reactions. It is found, however, that the more the bodies are purified, the fewer characteristic reactions of any kind do they show, and at the same time the more unstable do they become. This loss of activity of enzymes as they are purified may, probably, in some cases, be due to the removal of bodies necessary for the full activity of the enzyme, such as electrolytes, co-enzymes, and so forth. Amylase is inactive without neutral salts, the lipase of liver without bile-salts, pepsin without acid, etc. When this fact is known, it can, of course, be allowed for by the addition of the necessary co-enzyme, etc., after the process of purification has been performed.

The possibility must not be forgotten that a particular enzyme may be similar in its constitution to the substrate on which it acts, since there is such a close connection between certain enzymes and their own particular substrates, as we shall see later.

Perhaps the purest preparations known as yet are the invertase of W. A. Osborne [1899], the amylase of Fraenkel and Hamburg [1906], and the pepsin of Pekelharing [1902]. Let us see what the properties of these bodies are, leaving the method of preparation for the next chapter.

Enzymes not Proteins.

Osborne's invertase gave none of the protein reactions, except precipitation by copper sulphate, lead acetate, and phosphotungstic acid; it gave Millon's, the xanthoproteic and biuret tests very faintly. It was, therefore, not protein in nature. On the other hand, it was found impossible to free it completely from carbohydrate, which was afterwards identified by Koelle [1900] as mannose. According to Salkowski [1909] invertase contains no gum nor any other carbohydrate. So that, at present, it is not possible to express an opinion as to whether this is an essential component or not. It is suggestive that Fraenkel and Hamburg's amylase also contained a carbohydrate in small amount, but, in this case, a pentose. It also showed absence of typical protein reactions, that is, it gave neither the biuret nor the xanthoproteic reactions, but a faint indication of Millon's reaction.

The invertase could not be freed from ash, but the amount present was variable. The enzyme always contained nitrogen.

According to Euler, Lindberg, and Melander [1910], the purest preparations of invertase made by them contained mannose, but extraordinarily little nitrogen. The most active of them, in fact, only gave 0·36 per cent. of nitrogen (p. 162). This preparation was nearly twice as powerful as that of O'Sullivan and Tompson which contained 4·2 per cent. of nitrogen. In a further paper by Euler and Kullberg [1911], it is shown that the nitrogeneous constituent can be diffused away with gain in activity of the enzyme, weight for weight. Matthews and Glenn [1911], on the contrary, are inclined to regard invertase as a compound of a protein and a carbohydrate, as their preparations always gave a very faint biuret reaction and their activity was roughly proportional to the nitrogen content. The experiments of Euler, however, show that the nitrogen is not a necessary constituent.

Matthews and Glenn [1911, p. 51] propose a theory of the constitution of enzymes which is practically the same as that of Bertrand to be referred to presently. Enzymes are considered to be combinations of a colloid with an active principle. The former is related in nature to the substrate, while the latter, in the case of invertase and of diastase, is considered to be protein.

Přibram [1912] prepared malt diastase by a modification of the method of Fraenkel and Hamburg and regards it as composed of two substances, a polypeptide containing 7-8 per cent. of nitrogen, of comparatively simple composition, and a carbohydrate, which only reduces after hydrolysis. The proof is not given, however, that both these substances are essential to the activity of the enzyme.

Moreover, Beijerinck [1908] proved that an amylase was incapable of replacing either carbohydrate or nitrogeneous bodies in a nutritive medium for yeasts or bacteria.

Pekelharing's pepsin, in moderately concentrated solution, gave the majority of protein reactions, but contained no phosphorus, thus disposing of the view that enzymes are nucleo-proteins. The ash was very small, 0·1 per cent. The preparation was lævo-rotatory and very active, *viz.*, 0·001 milligramme in 6 c.c. of 0·2 per cent. HCl dissolved a flake of fibrin in less than twenty hours at 37° C. Perhaps its most interesting property is that it is relatively insoluble in water, but freely soluble in 0·2 per cent. hydrochloric acid.

On the whole it appears that all enzymes have not the same chemical structure, a fact probable enough in itself. Some indeed seem to belong to a class of bodies as yet unknown in chemical science, but containing nitrogen and carbohydrate in their molecules.

As further evidence on this point it may be mentioned that Rosenthaler [1909] found that emulsin is not precipitated by potassium

ferrocyanide and acetic acid, and that Hata [1909] found that enzymes are more readily precipitated by mercury salts than proteins are.

The purified enzymes referred to above were obtained in quite sufficiently concentrated solutions to give the typical protein reactions, if they were bodies of this nature. The statement made sometimes that enzymes can be detected by their action in solutions too weak to give the most delicate protein reaction, is therefore beside the point.

On the other hand, it is stated by Michaelis and Davidsohn [1911] that trypsin has the properties of the "α-nucleo-proteid" of Hammarsten, obtained from the pancreas. The optimal precipitation, or coagulation, occurs in very weak acid reaction ($H^{\cdot}$ ion concentration about $2{\cdot}6 \times 10^{-4}$); this is said to be so near the isoelectric point of trypsin ($H^{\cdot}$ ion concentration of $1{\cdot}35 \times 10^{-4}$) as to suggest that the precipitated substance is identical with the trypsin. It will be noticed that, nevertheless, the one of these values is double that of the other. The precipitate, in point of fact, had tryptic activities, but the clear liquid had also, so that it seems most probable that the trypsin was carried down by adsorption. Moreover, a similar precipitate obtained from extracts of pancreas devoid of trypsin, was itself devoid of tryptic action. The authors explain the absence of activity by the assumption of a slight chemical modification. It is also stated that no adsorbent would be capable of taking up so large an amount of trypsin. But how could the actual amount of the real enzyme be known? It was probably only a minute fraction of the total precipitate. It is further stated that the precipitated substance, when redissolved in weak alkali, gave no biuret reaction, a fact difficult to reconcile with its supposed protein nature. Further investigation is necessary with regard to trypsin.

Bertrand has advocated, since 1897, a view of the double constitution of enzymes. As stated in a lecture [1909] before the French Association for the Advancement of Science, it is as follows: One of the constituents is capable of producing, to a slight degree, on its own account, the chemical reaction associated with the particular enzyme in question, but requires its activity to be augmented by the presence of another substance, inactive in itself, before its action becomes appreciable. The former, according to the case, consists of acid, alkali, calcium or manganese salt, and so on. The latter component is a more complex substance, often similar to egg-white, colloidal in character. We shall find later more precise and definite evidence for the existence of systems of this kind. A view of the composite nature of enzymes, similar to that of Bertrand, is given by E. F. and H. E. Armstrong [1913, p. 566].

Enzymes as Properties.

When we consider the way in which definite chemical properties diminish more and more as the preparations are purified, we see a certain degree of justification for the view expressed by De Jager [1890] and by Arthus [1896], *viz.*, that enzymes are not chemical individuals, but that various kinds of bodies may have conferred upon them properties which cause them to behave like enzymes; so that we have to deal with properties rather than substances. The action, it is stated, can even be exerted at a distance. The experiments brought forward in support of this view are by no means convincing. I have myself repeated one of these, but was unable to obtain the result stated to happen. The experiment is of sufficient interest to warrant description. A solution of pepsin in hydrochloric acid is placed inside a parchment-paper tube and outside of this a solution of hydrochloric acid of the same strength. Cubes of boiled egg-white are then added to both solutions and the vessel left for some days at 38°. Pepsin itself cannot pass out of the tube, and yet, so it was stated, the egg-white in the outer fluid was digested. I found, on the contrary, that these cubes were absolutely intact, although the fluid gave a biuret reaction owing to the diffusion of the products of digestion of those in the inside of the tube. Possibly there may have been minute holes in the tube of the original experiment. A somewhat similar theory has been recently proposed by Barendrecht [1904], according to which enzymes act as radio-active bodies, the chemical activities of enzymes being due to radiations.

As already pointed out, inorganic catalysts and some organic ones are definite chemical compounds of known constitution, acting by the formation of intermediate compounds, also of known constitution. There is therefore justification for holding that colloidal organic catalysts, or enzymes, are also bodies of definite composition, at all events until stronger evidence has been brought to the contrary.

Effect of Heat.

Enzymes, as a rule, in contradistinction to inorganic catalysts, are destroyed by exposure to a temperature somewhere below 100° C., varying considerably according to circumstances. Some enzymes are more sensitive than others. This property is no doubt related to the colloidal nature of the enzyme itself or of some substance with which it is intimately connected. Recent researches, to be referred to in more detail in a later page, tend to show that this destruction by heat is by no means an essential property of an enzyme. Some enzymes, under certain conditions, are able to withstand the temperature of boiling water. Moreover, it appears probable that, in some cases at all events, the apparent destruction is due to some change in the other components of the complex colloidal system of which the enzyme forms a part. The action of heat cannot therefore be used as a reliable criterion in deciding as to the nature of a supposed enzyme. The catalytic properties are, in fact, the only satisfactory means of solving the question.

Distinction from Protoplasmic Action.

This effect of heat on enzymes obviously gives us no help in deciding whether an action is due to an enzyme or to the agency of living cells. Perhaps the distinction is at bottom one of words only, but at present there are many changes known to be produced by living cells of a kind such as no enzymes yet known are able to effect. Moreover, even if all cell-activities are due to enzymes, while the cells are growing there is a growth of enzymes also, so that the course of a reaction would be quite different in the two cases.

Antiseptics.

The use of antiseptics enables this distinction to be made. The life of protoplasm is impossible in the presence of an amount of antiseptic which has little or no effect on enzymes. At the same time it must be kept in mind that some enzymes are very sensitive to certain antiseptics, such as formaldehyde. On the whole, toluene appears to be the least injurious.

Filtration through porous clay or Berkefeld filters serves in some cases to exclude the presence of living cells, but since all colloids are more or less adsorbed by surfaces, considerable loss takes place until the filter is saturated with the enzyme.

New Production of Enzymes.

There is some evidence that an enzyme, previously absent, may be caused to make its appearance when required. Duclaux [1899] found that *Penicillium glaucum*, grown on calcium lactate, forms invertase only; grown on starch, it produces amylase in addition; grown on milk, it produces a proteoclastic enzyme. Abderhalden and Kapfberger [1910] state that the injection of egg-white, horse-serum, silk-peptone, gelatin, caseinogen, etc., caused the appearance in the plasma of an enzyme which hydrolysed glycyl-tyrosine and was not specific for the injected protein. The injection of lactose and other carbohydrates also causes the formation of enzymes which hydrolyse carbohydrates. This phenomenon is supposed to be a protective mechanism against the presence in the blood of foreign substances which cannot be utilised. The method used for the investigation of the proteoclastic enzymes was that called by Abderhalden 'the optical method,' which requires the detection of very small changes in rotation, and, considering the complex reactions taking place in serum when other proteins are introduced, it seems to me that confirmation of these results is necessary. It appears from previous work of the same investigator that the red corpuscles contain a proteoclastic enzyme, so that, admitting the correctness of the results, they may merely imply a certain amount of hæmolysis or increased permeability of the blood corpuscles. If this be so, there is no new formation of enzymes. Further discussion of these statements is beyond the scope of this book.

There is, however, a point of importance with regard to the chemical nature of enzymes which should be referred to here. The frequent occurrence of an enzyme capable of hydrolysing a substrate which it has never previously met with in the course of its evolution, such as lactase in almonds, or where it would produce toxic substances, as amygdalase in the alimentary canal of the vertebrates, means either that enzymes may be formed as it were accidentally, as bye-products of metabolism and having no relation to possible use, or that they are the opposite of specific, so that an enzyme which is useful for a certain purpose is also capable of acting on very different substrates on this account and cannot help doing so.

CHAPTER IV.

GENERAL METHODS OF PREPARATION AND OF INVESTIGATION.

Preparation.

It is not within the scope of this work to describe in detail the various methods used in the preparation of enzymes; for this, the original memoirs must be consulted. At the same time it will be useful to indicate the general principles on which these methods depend, especially as they throw light on the nature of enzymes.

Extractions from Cells.

Since the greater number of enzymes are found in cells, it is evident that they must be extracted by some means or other. Certain enzymes are found already in solution, so that this operation is unnecessary; such are pepsin in gastric juice, trypsin (or rather its zymogen) in pancreatic juice, erepsin in *Succus entericus*, and ptyalin in saliva. The juices of certain fruits, the pine-apple and that of the Papaw tree, for example, also contain proteoclastic enzymes.

When tissues or cells are extracted with water, best saturated with toluene or chloroform, it is found that some enzymes are extracted, others are not. In other words, the cell limiting-membrane is made sufficiently permeable by this means to allow such an enzyme as invertase to pass out; but even in this case better yields are obtained if the yeast-cells be allowed to disintegrate by autolysis. Many enzymes in fact cannot be obtained at all unless the cells are disintegrated; such are the zymase of yeast and the various autolytic enzymes of animal tissues. There are, accordingly, two chief classes of methods in use, according as the enzyme is "intracellular" or not. Lebedeff [1912], however, has shown that yeast, if dried in a certain way, will give up its zymase to water.

When mere extraction suffices it is sometimes advisable to use glycerol or weak alcohol in place of water, in order to avoid the deleterious effect of prolonged exposure to the latter; such a case is that of the extraction of trypsin, or rather trypsinogen, from the pancreatic cells.

Comparatively little disintegration seems requisite in the case of the enzymes found in the mucous membrane of the alimentary canal which hydrolyse disaccharides, since short grinding with sand and toluene water in a mortar suffices if the mixture be allowed to stand for some hours. The drying of yeast is efficacious for the extraction of similar enzymes from it, as was found by Emil Fischer [1894] and by Croft Hill [1898]. The yeast is spread out in thin layers on glass or porous plates, and dried as rapidly as possible in a current of warm and dry air; when dry, the yeast may, with advantage, be heated to 60° or 70°, whereby the subsequent extraction by water or weak alkali is facilitated, probably by more effective disintegration. In the dry state enzymes are much more resistant to heat than in the presence of water. The dried yeast obtained above may be kept for a considerable time without losing its properties as an enzyme.

More thorough disintegration is necessary in other cases, especially where the enzyme is "intracellular".[1] There are three chief methods for doing this.

Methods of Obtaining Tissue-Juice.

The first one is that used by Buchner [1903, p. 58], who was the earliest investigator to obtain the enzyme of alcoholic fermentation. It consists in thorough grinding with sand and kieselguhr in a mortar; the dry mass, or rather thick dough, thus obtained is then exposed to a pressure of 300 atmospheres in a hydraulic press. The kieselguhr is necessary, not only as a filter, but to afford a kind of support to the cell-contents so as to enable the great pressure to squeeze out the fluid from them. Of course the great surface of the kieselguhr adsorbs a considerable part of the enzyme, as shown by the fact that the press-cake from the first operation, by rubbing up again with saline solution and repeating the pressing, gives a further supply of the enzyme.

The cake sometimes contains the whole of the enzyme, as was found by Abderhalden and Pringsheim [1910] in the case of the proteoclastic enzyme of fungi. Probably the enzyme is insoluble in the tissue-juice, which is therefore inactive. Absence of enzyme from the press-juice from any tissue does not warrant the conclusion that the tissue contained none. Offringa [1910] gives some improvements and hints in the use of this method.

The second method is that of Rowland [1901], who used a kind of toothed wheel kept in very rapid rotation in the mixture of cells and sand. Apparently the grains of sand are driven with great force against

[1] For the general properties of this kind of enzymes see Vernon's *Intracellular Enzymes*. London, John Murray, 1908.

the cells and thus break them to pieces. A modification of this method, specially applicable to bacteria, consists in freezing the cells solid by means of liquid air and grinding them in this state in a steel mortar by means of a machine.[1]

The third, and most recent, method may be described as a development of the last. It is due to Wiechowski [1907], and was used by this observer in conjunction with Wiener [1907], to investigate the enzyme of the liver and kidney which destroys uric acid. The method is particularly valuable for animal tissues, and consists essentially in first reducing the tissue to a pulp by chopping finely and then pressing through a fine sieve to remove connective tissue, etc. The mass is then spread in thin layers on glass plates and dried as quickly as possible in a current of warmed and dried air. The dry film is then scraped off and ground to an extremely fine powder in toluene in a paint mill. The suspension is filtered by reduced pressure and washed with toluene to remove lipoid substances. On evaporation of the toluene a fine dry powder is left, which can be kept. To obtain solutions of the enzyme this powder is extracted with water, weak acid, or alkali, according to the properties of the enzyme in question, as regards its solubility, etc.

Purification.

Having by one or other of the methods described obtained a solution, which contains many other things in addition to the enzyme desired, the next step is to purify the latter as far as possible from these foreign substances. Inorganic salts and other diffusible bodies can be removed to a large extent by dialysis, as was done in the case of all three of the preparations of Osborne, of Pekelharing, and of Fraenkel and Hamburg mentioned above. It sometimes happens that the solution loses its activity to a greater or less degree during this operation. This is, in some cases, ascribed to diffusion through the parchment-paper and also to destruction of enzyme. There is, however, another possible cause, *viz.*, the removal of a co-enzyme, as we shall see in the eighth chapter, so that it is necessary to examine the diffusate before drawing conclusions.

[1] A more detailed account of these two methods is given in Harden's *Alcoholic Fermentation*.

Biological Method.

To remove proteins and certain carbohydrates, a very ingenious method was used by Effront [1899, p. 126] and improved upon by Fraenkel and Hamburg in their work on amylase. This may be called the "biological" method. As stated above, it was shown by Beijerinck that amylase is not a food-stuff for yeast, so that if solutions containing carbohydrate and protein are fermented with yeast, these bodies will be used up and the enzyme will be left behind. In the experiments of Fraenkel and Hamburg the yeast was made nitrogen-hungry by cultivating it in a nitrogen-poor medium. The method is certainly worth trying with other enzymes, especially with the proteoclastic ones, since, if these are proteins in their nature, they should serve as a source of nitrogen to micro-organisms. The converse does not necessarily follow. Enzymes may, of course, serve as nitrogen food, although not protein in nature.

I have, indeed, made some experiments of this kind with trypsin and found it incapable of serving as nitrogen food for yeast, but do not wish to lay much stress on these results except as tending to confirm other evidence of the non-protein nature of enzymes.

Precipitation.

Having purified an enzyme solution, by dialysis or otherwise, the enzyme is frequently precipitated by some means or other. Whatever the precipitant may be, prolonged contact with it is deleterious to the enzyme to a greater or less extent. Precipitation by acetone or alcohol and ether seems to be the most innocuous method, but in some cases, as in that of zymase and other intracellular enzymes, the precipitate must be filtered off from the alcohol as quickly as possible.

Saturation with ammonium sulphate is another useful method.

The carrying down of enzymes in adsorbed form with amorphous precipitates is an old method and sometimes is very serviceable. In order that this method may be an effective one, it follows, from what has been said in connection with colloidal adsorption-compounds, that the body to be precipitated and the precipitant should be electrically charged and also of opposite sign, unless an effective amount of electrolyte is present. Since most solid particles in water are electro-negative, the enzyme solution should be first made acid to render it electro-positive. Accordingly, we find that Michaelis and Rona [1906, 1907, 1 and 2], in their method of freeing liquids from proteins or of precipitation of enzymes by addition of mastic emulsion, first slightly acidulate the liquid, and some enzymes, *e.g.*, trypsin, require

in addition an electrolyte in the form of a salt for their precipitation. Jacoby's method of producing a precipitate of uranyl phosphate in an enzyme solution, which is on occasions a valuable one, comes under this head [1900, pp. 138 and 139].

Evaporation.

If it be desired to evaporate to dryness a solution of an enzyme, this must be carried out under reduced pressure and at as low a temperature as possible. For concentrating such a solution the apparatus of Schulze and Tollens [1] will be found useful. This consists in allowing the solution to trickle along an evacuated glass spiral, kept at 40° or lower. In this way the enzyme is only exposed to the raised temperature for a short time; a very effective evaporation is produced, as I can testify from experience.

Another useful method is that of Hladik [1910].

Investigation of Action.

In the investigation of the action of enzymes there are two different objects to be attained; the main problem may be the chemical nature of the bodies that are formed, or it may be desired to study the rate of change and the various conditions which affect it.

In the former case appropriate chemical methods are, of course, made use of, differing in each case. Although it is not the purpose of this short monograph to give details of the numerous chemical methods available, it is advisable to refer briefly to the method recently introduced by Sörensen [1907] on account of its theoretical interest. This method is devised for the estimation of the activity of proteoclastic enzymes and is based on the discovery of Schiff that, by the action of formaldehyde on amino-acids, the NH_2 group is neutralised by conversion into NCH_2. Formaldehyde is added in excess to a proteolytic digest, and the concentration of carboxyl groups can then be estimated in the usual way by titration with standard alkali. The increase in amino-acids and simple polypeptides during the course of the hydrolysis is determined by this means.

[1] See Lassar-Cohn, *Arbeits Methoden u.s.w.*, 4te Aufl., Allgem. Teil, p. 92, 1906.

Optical Activity.

In the latter case, since it is of importance to be able to make a large number of observations in a limited time, a physical method, such as that of *optical activity*, will be chosen when such a one is available. Whatever method be selected, it should, naturally, be that one which estimates that particular property which is subject to most change. For instance, in following the course of the hydrolysis of disaccharides, the polarimeter or the copper-reducing power is indicated. When the changes are not large, as in the investigation of reversion effects, Croft Hill's "optical factor," [1903, p. 583] gives valuable help; this is the ratio of the optical activity to the copper-reducing power, and since, in the production of a disaccharide from glucose, the former goes up and the latter down, the changes in the optical factor are larger than in either of the components alone. The method also tends to correct errors of estimation. As regards the determination of the *copper-reducing power* of sugars, the only really reliable method is that of Allihn in its various modifications. The standard method will be found in the work of Adrian Brown [1904, p. 78] referred to in the list at the end of this book, while the manner of preparing for sugar-estimation solutions containing enzymes is given in the paper by Aders Plimmer [1906, p. 23]. The method described by Bertrand [1906] is an accurate and useful modification of the gravimetric method. The cuprous oxide is estimated by dissolving in ferric sulphate and titrating the ferrous salt formed by standard permanganate.

The measurement of optical activity is also useful in the case of some proteoclastic enzymes, especially when acting on pure polypeptides; it has been used for this purpose by Fischer and Abderhalden [1905, p. 57] and by Euler [1907, **1**]. It is also available in the case of emulsin acting on glucosides. It has been used by Ring [1902] for the investigation of the action of pepsin and trypsin on proteins.

Refractive Index.

The *refractive index* may be *determined*, but, as a rule, its changes are small in the reactions which concern us here.

Spectro-photometer.

On the other hand, the colorimeter or the *spectro-photometer* has been made frequent use of. The oxidation of the leuco-base of malachite-green to the green pigment itself under the action of peroxidases, as investigated by Czyblarz and v. Fürth [1907], is a favourable case for such a method. The course of the change indicated by the biuret reaction has been followed by Klug [1895, p. 43] and by myself [1904, pp. 279 and 289] with the aid of the spectro-photometer. In using the method for colour tests certain difficulties have yet to be overcome, with regard to which the original papers must be consulted. It is possible that it may be made available for Millon's and the tryptophane colour reactions.

Viscosity.

Changes of *viscosity* are very marked in the action of proteoclastic enzymes on most proteins, which are, as a rule, in their unaltered condition, bodies with considerable viscosity (Spriggs [1902]). The method has its limitations, since it gives but little information as to the essential chemical work of the enzyme. This may be seen by comparing the change of viscosity with the nitrogen-content of the filtrate after precipitation by tannin, in the case of trypsin. It is found that for some time after the change in viscosity has come to a standstill, the production of peptone and amino-acids continues at a considerable rate [Bayliss, 1904, p. 15]. In other words, the reaction estimated by change of viscosity would appear to have come to an end, while it is actually proceeding at a fair rate. The same thing may be said about other similar changes in physical consistency, such as are at the basis of the methods of Grützner [1874] and of Vernon [1900-1, p. 406] with liquefaction of fibrin, of that of Fermi with liquefaction of gelatin and the various forms of Mett's tubes. After the fibrin flakes have disappeared, the protein will be still found to exist in a state which coagulates on boiling. As to gelatin, its capacity of setting when cooled is destroyed with extreme rapidity by trypsin. I found that five or six minutes' action of the enzyme at 40° was enough for the purpose, whereas very little chemical change had taken place in this time, as shown by the small amount of the change in electrical conductivity. The chief use of the above methods is to indicate the presence of a proteoclastic enzyme, or to compare the relative concentrations of two solutions of the same enzyme.

Dilatometer.

When changes of volume occur in the course of the reaction, *dilatometric* measurements may be made. The method, as applied to work with enzymes, may be found in Van't Hoff's paper [1910, p. 965].

Molecular Concentration.

Measurements of changes in molecular state would, in many cases, afford valuable indications of the work of the enzyme. Such may be obtained from *freezing-point* [Oker-Blom, 1902] or perhaps *vapour-pressure* determinations. The work of Benson and Wells [1910] on autolysis may be given as an instance of the use of a series of freezing-point determinations.

Gas Evolution.

When gas is evolved in the reaction, as in the case of zymase and glucose, the apparatus described by Harden, Thompson, and Young [1911] will be found useful.

Electrical Conductivity.

The last method to be referred to here is that of changes in *electrical conductivity*. Sjöqvist [1895], found that protein solutions showed a diminution of this property under the action of pepsin. The fact was confirmed by Oker-Blom [1902], who also showed that the action of trypsin was accompanied by a rise in conductivity and advocated the method as of great convenience. It has, notwithstanding, been hitherto used but little in the investigation of enzymes. Victor Henri and Larguier des Bancels [1903] have made a few observations with respect to the action of trypsin on gelatin, and I have myself made a somewhat extensive use of it for the general action of the same enzyme [1904, and 1907]. It showed itself to be of special value in this instance, since it was found to follow exactly the same time-course as the nitrogen in the tannic filtrate, the cause of the rise being almost entirely due to the production of peptones and amino-acids [1907]. The method may also be used for other proteoclastic enzymes acting in alkaline or neutral solutions. The change is more complex in acid solutions since the products of the reaction have a greater affinity for acid than the original substrate has; there is therefore at the beginning a fall of conductivity owing to diminution of hydrion. This subsequently gives place to a rise, but the exact course of the change has not yet been worked out.

The action of lipase on esters of the lower fatty acids may be fol-

lowed in this manner, since there is a considerable rise in conductivity owing to the formation of free acid.

In the hydrolysis of urea to ammonium carbonate by urease there is also a large increase in conductivity.

It is naturally not a good method for enzymes acting on carbohydrates, since there is, as a rule, no production of electrolytes in these reactions. An exception is the hydrolysis of sinigrin by myrosin, in which a good conductor, *viz.*, potassium hydrogen sulphate, is produced.

In the measurement of conductivity, the usual method of Kohlrausch may be used, but it is better to use Whetham's modification with rotating commutator [1900, p. 331], since there is no necessity in this method to use electrodes coated with platinum-black, which has a certain decomposing effect on many substances. The method has also the convenience that ordinary batteries and galvanometer are used.

Detection of Trypsin.

In order to detect the presence of trypsin, peptone "Roche," a product of partial hydrolysis of silk, is very valuable [Abderhalden and Schittenhelm, 1909]. This polypeptide contains a large amount of tyrosine, which is separated off by tryptic enzymes but not by pepsin. Tyrosine, being comparatively insoluble, rapidly crystallises out. See also Abderhalden and Steinbeck [1910, 2] and part II of Plimmer's monograph in this series.

Estimation of Strength.

When comparing the action of different strengths of enzyme solutions, it is advisable to take as the basis of comparison the times taken to effect an equal change, rather than the amounts of change in equal times. This is especially important where the reaction takes place in stages, since only in this manner is it possible to have comparable values. Instances of such reactions are the action of amylase on starch and proteoclastic actions in general. If the reaction has not arrived at the same stage in the cases to be compared, there is a source of error due to the various stages not being passed through with the same velocity.

In the case of trypsin, for example, proteoses are produced very rapidly and are again converted fairly rapidly into peptone, while this latter body is, in part, hydrolysed further but more slowly into amino-acids ; at the same time some amino-acids are produced from the first, so that the reaction is a very complicated one, and the results obtained at different stages would be very difficult to interpret, unless times of equal change are chosen for comparison.

Methods of Stopping Action.

Another practical question may be touched upon to conclude this chapter. When the reaction has to be stopped at a given stage for the purpose of determining how far it has gone, as is necessary in the use of most of the methods described, with the exception of that of electrical conductivity or, in some cases, the optical activity, one must be able to stop the action of the enzyme by some means. Now it is by no means a matter of indifference how this is done. As will be shown in detail in a later chapter, the activity of enzymes is enormously increased by rise of temperature, although in the end abolished. The method of taking a certain volume of the reacting mixture and heating this to boiling-point, or in a steam steriliser, as is frequently done, is liable to be, and in fact has been, the source of errors. It is plain that the solution cannot be heated instantaneously to the destruction temperature of the enzyme, and, during the time taken for this to be attained, the activity of the enzyme is enormously increased. In this way changes take place after the action is supposed to have ceased. If it is requisite that the action should be stopped by heat, the solution may be run in a thin stream into boiling water. If not desirable to dilute the mixture, it may be cooled to 0°, or better, frozen solid and kept so until wanted for investigation. When the addition of chemical reagents is immaterial, this means of stopping the action of an enzyme is most convenient. Ammonia may be added to invertase experiments; in this case it is of use in another way, in that it gets rid of the bi-rotation of glucose, by bringing the glucose system to an equilibrium at once. Alkali may be added to pepsin or acid to trypsin. Of course when a precipitant, such as tannin or phosphotungstic acid, is used in the further stages of the method it may be added at once to stop the action.

It may be noted that the advantage of the electrical conductivity method is that readings can be taken, by using appropriate vessels, without disturbing the course of the reaction. The same thing applies to the viscosity method and to the polarimeter method, if precautions are taken to keep the solution at a constant temperature, in the latter case by a jacketed tube through which water is flowing.

Nothing has been said as to the maintenance of a constant temperature in following the course of enzyme action, important as it is. The best means of doing this can easily be found by consulting such a work as that of Ostwald and Luther on physico-chemical measurements.

CHAPTER V.

REVERSIBILITY OF ENZYME ACTION.

REFERENCE has already been made in the first chapter to the fact that in the case of such a reaction as that resulting in the equilibrium between methyl acetate, acetic acid, methyl alcohol and water, both the hydrolysis of the ester and also its formation from the acid and alcohol are accelerated by a catalyst such as hydrochloric acid. If therefore enzymes are to fall into line with other catalysts, they will also accelerate synthetic processes.

This is a matter of sufficient importance to warrant repetition. If enzymes are catalysts and if the reaction accelerated, or brought about, by them is a reversible one, the same agent which produces hydrolysis must also produce synthesis. In any particular case, therefore, where we find a definite equilibrium position anywhere but at that point which corresponds to complete change in one direction, the enzyme acting is producing both hydrolysis and synthesis. Moreover, when, as is actually the case, the reaction is brought to an end at the same position, not only by different concentrations of the same preparation, but also by various preparations, treated by reagents which partially destroy it and so forth, it is impossible to believe that two enzymes, one hydrolysing only, the other synthesising only, should always be found in exactly the same relative concentration.

These considerations apply, of course, only to reversible reactions. But, as J. J. Thomson points out [1888, p. 281], all reactions are theoretically reversible and the apparent irreversibility of some is merely due to the limitation of our own powers of manipulation, such as appropriate temperature, etc. Statements of a similar nature will be found in the book by Nernst [1911, p. 442].

We know that many processes of the kind known as reversible, or balanced, reactions take place in the living organism. Particularly obvious are those cases where material is stored up in an insoluble form, like starch or glycogen. These bodies are, under certain conditions, synthesised from sugars and, under other conditions, are hydrolysed back again, when required.

Since the first definite proof of a synthetic process taking place under the influence of an enzyme was brought forward by Croft Hill [1898], so many other cases have been discovered that it is no longer necessary to give a list of them. In fact the impression is distinctly given that it is merely a question of finding the proper conditions in order to be able to obtain synthesis from all enzymes.

Effect of Water.

As regards these conditions the first thing that requires attention is the part played by water. Methyl acetate can be kept indefinitely in a closed bottle without change, but the presence of the smallest amount of water causes the hydrolysis of a part of it, and the greater the proportion of water, the more the reaction takes place in the direction of the formation of acetic acid and alcohol. Stated in another way, the greater the relative concentration of the water component, the nearer the equilibrium-point is to the position of complete hydrolysis.

Since enzymes are colloids, the reactions in which they take part occur in heterogeneous systems of, at least, two components. The enzyme-phase contains less water than the solution of the substrate, so that, if the bodies to be synthesised should happen to be more soluble in the enzyme-phase than in water, not only will their partition be in favour of greater concentration in the enzyme-phase, but, water being nearly absent from this phase, synthesis will be further accelerated. Surface-condensation or adsorption by the enzyme will have the same effect.

Synthesis by Invertase.

Amongst enzymes there are many instances where the hydrolysis appears to be complete, invertase for example. But it has been shown by Visser [1905, p. 275] that a 0·25N solution of saccharose gave only a rotation of $-3·26°$ when acted on by invertase until no further change took place, whereas when inverted by acid the final rotation was $-3·420$, which is what the reading should be if the solution contained only glucose and fructose. And again, a particular solution containing equal amounts of glucose and fructose had an initial rotation of $-12·46°$; after the action of invertase for two months the rotation had fallen to $-12·29°$. A change of this degree means that an equilibrium position exists when about 99 per cent. of the saccharose is hydrolysed, and that, if the products, glucose and fructose, be exposed to the enzyme, a formation of saccharose to this extent takes place. When we remember that the equilibrium position is given by the ratio of the velocity of the hydrolytic to that of the synthetic process, we see at once how very

much the former exceeds the latter. Visser in fact found that, for 0·5N saccharose, the equilibrium-constant, *i.e.*, the ratio of the two velocity-constants, was very nearly 50, so that, since six days are required to attain equilibrium when saccharose of this concentration is acted upon by invertase, about ten months (*i.e.*, fifty times six days) would be needed for the reverse reaction [1905, p. 301].

Importance of Small Amount of Synthesis.

Now it might be thought that a synthesis of so small a degree could not be of much practical importance. This would be an error, as the following considerations will show. Let us take the case of amylase where a similar reaction, progressing almost to completion, occurs, and let us suppose that no more than 1 per cent. of starch is formed when the enzyme acts upon maltose or dextrin. Since the product is an insoluble body the equilibrium will exist only for a moment, so that more starch will be formed in order to replace that thrown out of the system by precipitation. As the rate of this reaction is slow, as shown above, the amount of starch formed per unit time will not be great, although by no means negligible. The process, it will be noted, is analogous to that of the precipitation of chloride as silver salt. It is most likely, as Croft Hill points out, that the storage of starch in the plant and that of glycogen in the animal are to be explained on these lines 1898, and 1902].

The hydrolysis and loss of starch from germinating seeds is regulated by the growing plant. If the embryo is removed, the starch ceases to be hydrolysed. This seems to be a case of equilibrium in a reversible reaction, since, as Pfeffer and Hansteen [1893] have shown, if the embryo of maize or barley be replaced by a little column of plaster of Paris, the disappearance of starch can be stopped or set going according as the end of the plaster column is immersed in a tiny drop of water or a large quantity. In the former case, the products of hydrolysis are not removed, so that the reaction comes to an equilibrium. In the latter case, they are removed by diffusion as fast as they are formed, so that their concentration is maintained permanently low and no equilibrium is reached.

Moreover, it is not necessary, in order that considerable synthesis may take place when the equilibrium-point is close to that of complete hydrolysis, that the synthetic product should be deposited in an insoluble form; it may be removed from the reacting system by any other means, such as diffusion into blood-current or elsewhere, or taken up in some other independent reaction.

Synthesis by Lipase.

The simplest case of reversibility is that of lipase acting on esters of lower fatty acids, which was first investigated by Kastle and Loevenhart [1900]; we will therefore briefly examine this reaction. It may perhaps seem strange that the action of maltase on glucose, in which the synthetic action of enzymes was first discovered by Croft Hill, has not yet been dealt with. The reason for this omission is that the conditions here have turned out to be complicated by the existence of the two optical isomers of the bi-hexose which is formed, so that the reaction will best be discussed at a later stage.

It is quite easy to observe the production of ethyl butyrate when lipase acts on a mixture of ethyl alcohol and butyric acid, since the ester has a characteristic odour, very different from the acid or alcohol. To quote the authors named : " When a fresh aqueous extract of pancreas is treated with a mixture of dilute butyric acid (0·1 to 0·05N) and ethyl alcohol (sufficient to bring the whole to 1·5 per cent.) the very characteristic odour of ethyl butyrate soon develops even at the ordinary temperature and in the presence of antiseptics, whereas if the pancreatic extract is first boiled the mixture never develops the odour of the ester ". If the experiment be done on a large scale, the ester can be separated by distillation and can be hydrolysed back again by the same enzyme that produced it. Moreover, when ethyl butyrate is hydrolysed by lipase, the reaction is never complete, so that, in other words, an equilibrium is arrived at. It is of interest that the hydrolysis does not proceed so far when effected by lipase as when effected by hydrochloric acid, as has been recently shown by Dietz [1907, p. 320]. As was pointed out in the first chapter of this book, this circumstance does not mean that the enzyme does not follow the general laws of catalysis, but that its mode of action is by the formation of some kind of compound between enzyme and ester on the one side and between enzyme and products on the other side, or, more probably, that the equilibrium position is altered by surface energy (see pp. 7 and 63).

Now pancreatic lipase hydrolyses the higher fats as well as the simpler esters ; synthetic production of higher esters of glycerol would therefore be expected to take place and has been actually observed ; in fact, Hanriot [1901] has obtained by means of lipase a butyric ester of glycerol, monobutyrin, and Pottevin has obtained mono- and tri-olein [1903 and 1906].

The physiological importance of this reversibility of lipase-action is pointed out by Loevenhart [1902] himself. In the process of digestion and absorption of fat there is no doubt that fat globules are found in

the cells of the intestinal mucous membrane and that fat taken as food is hydrolysed in the lumen of the intestine. There must therefore be some mechanism by which the products of hydrolysis are resynthesised in the cells after absorption. It is obvious that if a lipase were present in these cells, it would be capable of considerable synthetic action, since the fat produced, being insoluble, is deposited out of the reacting system in the form of droplets. Loevenhart has in point of fact been able to obtain a lipase from intestinal mucous membrane of the pig after thoroughly washing away the pancreatic enzymes. A similar enzyme was also obtained from the liver and other places where fat storage occurs. In all these cases, when the blood and lymph bathing the cells becomes poor in fatty acid and glycerol, either owing to fat being stored elsewhere or to its being used up by oxidation, as in starvation, the lipase restores equilibrium by effecting hydrolysis of the fat which had previously been stored up.

Bradley [1913, 1] has compared the lipase content of various tissues with the amount of fat contained therein, and finds that there is no parallelism between the two. He comes to the conclusion that no support is given by these experiments to the view that the fat is synthesised by the enzyme. On the other hand, they do not negative the hypothesis. The amount of enzyme present does not affect the equilibrium position, merely the rate of the reaction, and, in most cases, fat is laid on slowly as a storage product, so that rate of production is not a matter of great importance. The active mammary gland is, undoubtedly, a difficulty, since it contains less lipase than the spleen and the brain. We shall find the same difficulty in the case of the lactose of the milk. It is very probable that the formation of milk is a secretory process in which enzymes play no part. Synthesis is by no means always a result of catalytic action. Methyl glucoside, for example, is produced by the action of alkali on methyl sulphate in the presence of glucose. With regard to the general question, it must not be forgotten that there is free communication in higher animals between all organs, effected by means of the blood, so that enzymic synthesis may be carried on in one place and the product stored up or excreted in another place. In fact, it is quite conceivable that this may be the more effective method, owing to the removal of the products allowing continuous synthesis to proceed.

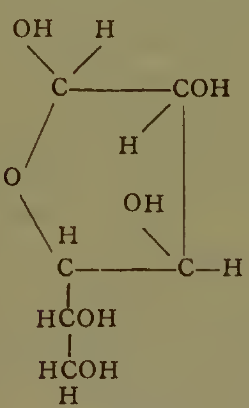
It is stated by Thiele [1913, p. 296] that the liver, spleen, kidney, and muscle contain a lipase which can hydrolyse lecithin only, having no action on ordinary fats. His experiments, however, merely show that, in the time allowed, the lecithin in a mixture with fats was attacked before the latter. In the pancreas the lipase was present in

sufficient amount to attack both. No experiment is given with a pure emulsion of fats without lecithin. An experiment on autolysis at 4° is given, no hydrolysis was found; none would indeed be expected at so low a temperature.

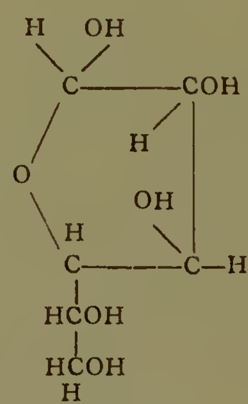
Another interesting case is the formation and destruction of fat in the larva of Calliphora. According to the investigations of Weinland [1909], this process is governed by an equilibrium condition under the action of enzymes.

Synthesis of Carbohydrates and Glucosides.

Before proceeding to the more difficult cases of synthetic action on carbohydrates, it is necessary to say a few words about the stereochemistry of the glucosides. These bodies are recognised as having the internal anhydride structure of a γ-lactone. Glucose itself has the same structure and is therefore capable of existence in two optically isomeric forms, known as α- and β-glucose, which differ from one another in the relative positions of the H and OH of the terminal aldehyde group as shown in the formulæ:—



α-glucose.



β-glucose.

One of these two forms, which we call, for convenience, the α-form, has a greater specific rotation than the β-form. In glucose in the dry state the first preponderates; when dissolved in water the rotation is at first higher than after standing, and it decreases until a state of equilibrium is finally established. According to Tanret, in a 10 per cent. solution in equilibrium there exists 3·7 per cent. of the α-form and 6·3 per cent. of the β-form. It was found by Emil Fischer [1898-9] that, when a solution of glucose in methyl alcohol, in which presumably both forms of the sugar are present, is acted on by hydrochloric acid, two methyl-glucosides are formed. One of these, the α-form, corresponds to the left-hand formula of the two above, in which the hydrogen of the uppermost OH is replaced by CH_3, while the β-methyl-glucoside is similarly formed from the other.

For further details as to the stereo-chemistry of the glucosides the reader is referred to the monograph by Frankland Armstrong in this series.

In all yeasts which are able to ferment maltose, as shown by Fischer, there is present an enzyme which is able to hydrolyse the maltose as a necessary preliminary to the action of zymase, which does not act upon bi-hexoses. The enzyme hydrolysing maltose is known as maltase and is not identical with invertase. Now it is found that maltase will hydrolyse the α-methyl-glucoside, but not the β-form, whereas emulsin, the enzyme found in bitter-almonds and elsewhere, which acts upon most of the natural glucosides, such as amygdalin, salicin, etc., will not touch the α-glucoside, but readily hydrolyses the β-form. The conclusion is that maltose has the structure of an α-glucoside and that the natural glucosides, such as salicin, are β-glucosides.

If maltase be allowed to act upon a strong solution of glucose we should expect that, since this enzyme hydrolyses maltose to glucose, if any synthetic process takes place, maltose would be formed, and this was in fact what Croft Hill [1898] at first believed to take place. It turned out, however, on separating the bi-hexose which was produced, that it consisted only partially of maltose, the rest being, as supposed, a new sugar, revertose [1903]. Emmerling [1901] showed later that this "revertose" was actually isomaltose, and Frankland Armstrong [1905, p. 598] has confirmed this. What then is isomaltose? It is a bi-hexose which is hydrolysed by emulsin with the production of two molecules of glucose, so that we are justified in regarding it as the optical isomer of maltose, *viz.*, the β-glucose-glucoside. Now according to the hypothesis first put forward by van't Hoff [1898, p. 12] and accepted by Croft Hill, an enzyme only synthesises the same body which under other conditions it hydrolyses. Moreover, this is the only possible hypothesis if enzymes are to be brought into line with other catalysts. It is therefore of some importance to examine more closely the facts as far as we know them. Isomaltose is not hydrolysed by maltase, but by emulsin; it is therefore, to begin with, rather startling and suggestive that, in Croft Hill's experiments, the synthetic products are almost completely hydrolysed back by the same enzyme preparation that produced them. For example, a 45 per cent. solution of glucose was acted on by yeast extract until its "optical factor" (see earlier, Chapter IV.) had been raised from 0·525 to 0·676, which indicates a synthesis of 15 per cent. reckoned as maltose. After boiling, 10 c.c. of this solution was diluted to 200 c.c., some of the original yeast extract was added and it was allowed to stand at 28° for ten days. The optical factor was now reduced to 0·537, indicating almost com-

plete hydrolysis, while a control with the same amount of boiled enzyme solution showed only a reduction to 0·675 [1903, p. 585]. Further, it was found that if the synthetical products were fermented by a pure maltase-containing yeast, such as *Saccharomyces Ellipsoideus I.*, only a part of the bi-hexose was hydrolysed and fermented, presumably the maltose alone. It seems to me that the explanation of the production of isomaltose lies in these facts. In the first place it is to be noted that the enzyme solutions used were made from ordinary brewer's yeast, and that these extracts hydrolysed practically the whole of the synthetical products, whereas pure maltase only acted on a part. Croft Hill himself suggested the presence of a mixture of enzymes in his extracts, and since that time it has been shown that many yeasts contain emulsin (Henry and Auld [1905]); in fact ordinary pressed-yeast will rapidly hydrolyse amygdalin at a temperature of 38°, as can easily be verified. The conclusion to be drawn is that the formation of isomaltose can be satisfactorily explained as being due to emulsin and that of maltose by the presence of maltase.

Again, Fischer and Armstrong [1902] found that, by the action of Kefir-lactase on a mixture of glucose and galactose, isolactose and not lactose was formed. On referring to the table given on p. 3151 of their paper, the significant fact will be noticed that this synthetic product was hydrolysed, in dilute solution, by the same enzyme, or mixture of enzymes (Kefir-lactase), that produced it under other conditions. It is clear, therefore, that the enzyme preparation contained a body capable of hydrolysing isolactose, and there is no reason to suppose that the synthesis was effected under the influence of any other enzyme.

A quite different point of view has been taken by Frankland Armstrong [1905], who regards it as the rule that an enzyme synthesises exactly those bodies which it does not hydrolyse. Now it seems to me that such a view is calculated only to throw the whole subject into confusion; but, apart from this, there are many reasons for not accepting it. It was shown by Croft Hill that in the action of maltase on glucose an equilibrium-point was reached, in the conditions under which he worked, when about 15 per cent. of the glucose had been synthesised to the bi-hexose and that the reaction then ceased. If the synthetic body were incapable of hydrolysis by the enzymes present, there is no conceivable reason why the reaction should cease until the whole of the glucose is converted, because, as the synthetic glucoside is not acted upon, it is withdrawn from the sphere of action as soon as it is formed. Granting that the body isolated by Frankland Armstrong from the action of yeast extract on glucose was isomaltose, it

must be remembered that the yeast used was ordinary brewer's yeast, which was shown by Henry and Auld to contain emulsin. It will also be noticed that the yield was small, and the possibility that the same enzyme may act on both optical isomers, but at different rates, as suggested by Fajans [1910], is not to be forgotten. In fact, it seems to me to be the most probable explanation of the results in question, as well as of some other apparently anomalous cases. It is interesting to note, in confirmation of this view, that Dox and Neidig [1912] have shown that living *Aspergillus*, as also extracts of the mycelium, will hydrolyse both α- and β-methyl-glucosides, but the former only at about one-sixteenth the rate of the latter.

In his monograph in this series (p. 75), Frankland Armstrong objects to my interpretation of his results that I have not taken into account the fact that the equilibrium will be affected by the combination of enzyme with sugar. It does not seem to me to be sufficient to explain the fact that the synthesis ceases when 15 per cent. of glucose is converted into a bi-hexose, as in Croft Hill's experiments. The retarding influence of glucose and the loss and strength of the enzyme should merely delay the attainment of the equilibrium. The dilution caused by the conversion of the whole of the glucose into disaccharide would do no more than change the initial concentration of 45 per cent. into one of 42·7 per cent., so that when 15 per cent. was converted the change in carbohydrate concentration would only be from 45 per cent. to 44·65 per cent. I quite agree, however, that further experimental investigation is much wanted.

My explanation of the results of experiments on synthesis of bi-hexoses may also be objected to for the reason that the amount of maltose found by Croft Hill was much less than that of "revertose" or isomaltose. The cause of this fact is no doubt that given by the experimenter himself, *viz.*, that the yeast used contained amylase or dextrinase, under the synthetic action of which the maltose was partially converted into dextrin; bodies of this nature did in fact make their appearance in the process of separating the products.

It has been pointed out by Fajans [1910] that if we assume that the same enzyme may hydrolyse both maltose and isomaltose, but that its action on the latter is less powerful and takes place at a slower rate, the explanation of the synthesis of optically active bodies is simple, when the enzyme itself is also optically active.[1] When acting synthetically, it is not unlikely that the enzyme may cause the production of isomaltose more rapidly than it does that of the isomer, maltose, so that in equilibrium the former will preponderate. It may even happen

[1] For further details see note E, "On the Specificity of Enzymes".

that the two isomers are formed at the same rate, but since, by hypothesis, maltose is hydrolysed more rapidly than isomaltose, it is plain that in equilibrium the concentration of the latter will exceed that of the former.

The recent work of three investigators on the synthesis of glucosides by emulsin requires attention here. Van't Hoff [1910] points out that, according to the researches of Menschutkin on esterification, it would be expected that glucosides of primary alcohols would be much more easily synthesised than those of secondary alcohols, and these in turn more easily than those of tertiary alcohols. I have been able to confirm this directly in the case of the glucosides of isoamyl alcohol (primary) and the corresponding tertiary alcohol, dimethyl-ethyl-carbinol [1913, p. 250]. Accordingly, even in concentrated solution, an equilibrium-point corresponding to the presence of more than 5-10 per cent. of the glucoside is not to be expected in the case of such naturally occurring glucosides as salicin, arbutin, etc., which are compounds of substances having the structure of tertiary alcohols. Dilatometer experiments were made in order to test whether this equilibrium-point actually could be shown to be near the position of complete hydrolysis. The results showed this to be the case, although the experiments were not made in such a way as to exclude the possibility of a small amount of synthesis, and van't Hoff himself notes (p. 966, footnote) that a slow change in the direction of synthesis was observed in an experiment which was allowed to proceed for a long time. I do not quite understand the statement of E. F. Armstrong [1912, p. 102] that these experiments altogether exclude the possibility of an equilibrium in a reversible system. The question will be found further treated in my paper on synthesis by enzymes [1913, pp. 252-54]. When, on the contrary, a primary alcohol, glycerol, was taken by van't Hoff, he found it easy to obtain a synthesis of 60-70 per cent. of the glucoside. I also came across this fact, before becoming acquainted with van't Hoff's work, and have made use of the reaction to test various questions, as will be seen later. In this place it is sufficient to remark that the synthetic glucoside is the β-form and identical with that which is hydrolysed by emulsin.

A large number of the β-glucosides of various primary alcohols have been made by Bourquelot and Bridel [1911-13] with the aid of emulsin, and several important questions connected with the action of enzymes have been elucidated. These glucosides have been isolated in the crystalline state and their identity satisfactorily established; so that the objection of E. F. Armstrong [1912, p. 102] that the synthetic products had not been isolated and identified, is answered.

The conclusion we come to is, then, that there is no cogent evidence that enzymes produce by synthesis any bodies different from those which they hydrolyse.

Bradley and Kellersberger [1913, 1 and 2] have investigated the diastase (glycogenase) of animal tissues in comparison with their glycogen content, and state that there is no parallelism between them. Tissues containing most enzyme do not in fact all contain glycogen, while some of those rich in glycogen do not contain enzyme. Owing to the chemical communication between various animal organs by means of the blood, as already mentioned in connection with lipase, the experiments, while adding nothing in support of the view that synthesis in tissues is effected by the agency of enzymes, do not disprove it. On the other hand, it was found that no plant tissue containing starch was devoid of amylase, although some tissues contained enzyme without starch. It will be noted that intercommunication between the tissues of plants is not so free as in animals.

The absence of lipase from the mammary gland, as shown by Bradley, was referred to in the previous section of this chapter. The same observer [1913, 2] finds that lactase, also, is absent from this tissue. The formation of lactose appears, therefore, to be a protoplasmic activity independent of an enzyme, unless the lactose is formed elsewhere and transported to the mammary gland. The whole question of the formation of the constituents of milk is as yet an unsolved problem.[1]

"Synthetic Enzymes."

The suggestion has been made that synthetic processes are brought about by special enzymes, which do not hydrolyse. As yet no enzyme having this property has been prepared. We have seen, moreover, that the hypothesis of the existence of such bodies is unnecessary in the present state of our knowledge, and, therefore, according to the canons of scientific method, must be rejected. It is difficult also to reconcile such a view with the facts concerning the equilibrium-position. In all the experiments in which attention was directed to this point, such as those of Croft Hill, Visser, Dietz, Bourquelot, and myself, it was found that this equilibrium-point was the same under the same conditions of concentration, etc., whatever the amount of the enzyme used, and that different preparations also gave the same result. If the enzyme preparation were a mixture of a hydrolysing and a synthesising enzyme, it seems extremely unlikely that the two bodies should always be

[1] For a summary of the question of carbohydrate synthesis in plants, the reader may consult the article by Prof. Priestley in *Science Progress*, July, 1913.

present in the same relative proportions. Again, it is found that synthesis takes place chiefly under conditions in which, according to the manner of reversible reactions, it would be expected to occur, *viz.*, high concentration of the products of hydrolysis.

Rosenthaler, however [1908], claims to have separated from emulsin an enzyme which hydrolyses, but does not synthesise, benzaldehyde-cyanhydrin. The description of his experiments is too meagre to enable any conclusion to be drawn in a case of so much difficulty. It appears that by half saturation with ammonium sulphate, a precipitate is produced in solutions of emulsin. The filtrate from this is stated to possess hydrolysing properties only, while the precipitate redissolved has synthetic power in addition to the hydrolytic one. It could not be washed free from this hypothetical hydrolysing enzyme. If, as it seems, the action of these two components of emulsin was tested, in the one case in the presence of half saturated ammonium sulphate and in the other in watery solution, it is obvious that the conditions were not the same. Copper sulphate, also, caused separation of the two bodies; they were also destroyed by heat at different temperatures.

I have repeated these experiments [1913, pp. 254-57], using the synthesis of glycerol-glucoside as a test reaction, since it is much more easily worked with than that used by Rosenthaler and not so liable to cause confusion. The presence of an enzyme in the filtrate which apparently hydrolyses only was found to be due to partial precipitation or destruction of the single enzyme by the various kinds of treatment referred to above. Hydrolytic power is easily to be detected, whereas synthetic activity requires a more concentrated solution of the enzyme in order that it may be obvious in a reasonable space of time. Synthesis was quite readily detected if the very dilute solutions, obtained by Rosenthaler's various procedures, were concentrated at a low temperature under reduced pressure. As my experiments were made in the presence of excess of glycerol, I added a little glycerol before evaporating the solutions and thus obtained a concentrated solution of the enzyme in glycerol.

Further, according to Rosenthaler's view, the equilibrium attained under the action of emulsin must be due to the presence of both a synthesising and a hydrolysing enzyme in certain proportion to one another; otherwise, the reaction would be complete in one or the other direction. Now, according to the same observer, these two enzymes are not equally sensitive to the action of heat. Therefore, if a preparation which has been warmed for some time, and lost part of its activity, be allowed to act on a mixture of glucose and glycerol,

the equilibrium-position cannot be the same as that under the action of the same enzyme before exposure to heat. In point of fact, I found [1913, p. 257] it to be identical in the two cases, although, after warming the enzyme solution, the time taken is naturally prolonged, owing to partial destruction of the emulsin, as Tammann showed. The reader is also referred to Note E for other factors to be taken into consideration.

A somewhat peculiar position is taken by Euler [1907, 2 and 1907, 3] on the strength of an experiment of Beitzke and Neuberg [1906], who showed that on subcutaneous injection of emulsin into a rabbit, anti-emulsin was formed and that this body was able to synthesise lactose from glucose and galactose.

Beitzke and Neuberg themselves do not claim that the effect they obtained is capable of generalisation, since they found that anti-lipase, which was formed by subcutaneous injection of lipase, had no synthetic action. Moreover, the synthetic action of anti-emulsin itself has been called in question by Coca [1907]. In any case it seems premature to assume, as Euler has done, that anti-enzymes in general have synthetic powers. The effect of such hasty assumptions may be in certain cases of a misleading nature and tend to retard the progress of real knowledge. Only confusion can result from the formation of general laws on the strength of isolated cases.

In fact, on repeating these experiments [1912], I have been compelled to come to the conclusion that no anti-emulsin is formed when emulsin is injected into rabbits; there is indeed the production of a precipitin for the foreign protein present as impurity in the enzyme solution. This anti-body does not affect the enzyme itself, which is left in the solution when the precipitin is added to it. The fact is of interest as confirming the conclusion we had already arrived at, that enzymes are not proteins. It may also, perhaps, be made use of to separate the protein constituents from the actual enzyme in some cases.

The serum from rabbits "immunised" against emulsin has no greater inhibitory action on the enzyme than normal serum has, as Coca [1907] pointed out. My experiments show that when there is any action of this kind it can be explained as being due to diminution of the $H^{\cdot}$ ion concentration of the solution to which serum is added. If the reaction is brought back to its former value, the activity of the enzyme is unchanged. Moreover, if an emulsin solution be brought to the same $H^{\cdot}$ ion concentration as that produced by addition of "immune serum," the reduction of activity is the same. The appropriate method of producing these changes of reaction is by the addition of acid and alkaline phosphate in definite proportions. The anti-emulsin is supposed to be

contained in the globulin fraction of the immune serum. I found that this substance had no effect whatever on the activity of the enzyme, and, in fact, it had no action on the H· ion concentration.

It is scarcely necessary to add that I could obtain no evidence of the synthesis of lactose. The evidence brought forward by Beitzke and Neuberg is very inconclusive, and in a later paper [1909] they suggest that the effect was due, not to an anti-enzyme, but to the presence of Rosenthaler's synthesising emulsin, which was supposed to have remained unchanged for a longer time in the organism than the hydrolysing form, whose absence from the blood they believe that they were able to show. I have already pointed out that the existence of such a substance is merely imaginary.

Apart from the unsatisfactory experimental evidence, it is worth directing attention to the fact that the attribution to an anti-body of chemical activities opposite to those of its antigen is contrary to the meaning of "anti-body" in Ehrlich's sense. An anti-body produces its effect by neutralisation, by precipitation or otherwise, of the antigen which gave rise to its formation. For example, the anti-body formed in response to the injection of a foreign protein is a substance which precipitates this protein from its solutions; and, in this case, is therefore called a "precipitin". A true anti-emulsin would be a substance produced in the blood in consequence of the injection of emulsin, which acts on the enzyme, its "antigen," in such a way as to render it inactive. When emulsin hydrolyses lactose, its true anti-body is not a substance that synthesises lactose, but one that prevents emulsin from hydrolysing it, which is by no means the same thing. We have seen above that the same enzyme causes either synthesis or hydrolysis, according to which side of the equilibrium position the system happens to be on when the enzyme is added to it; if the original view of Beitzke and Neuberg, adopted by Euler, were correct, the same chemical individual is anti-body to itself as antigen.

In this connection, Euler's statement [1911] that there is an enzyme in certain extracts of yeast which synthesises hexose-phosphate, but does not hydrolyse it, may be referred to. It was, in fact, to meet this case that the termination "ese" for such enzymes was proposed. Harden and Young [1908], however, had already described an enzyme in very similar extracts which hydrolyses hexose-phosphate. The conditions are too complex, however, to warrant the introduction of a new name, especially when it assumes that enzymes do not obey the laws of catalysis. Until further evidence is produced, there is no reason why both functions should not be given to the same enzyme,

as I have shown must be done if enzymes are catalysts. The necessity for a new name accordingly disappears.

With respect to these hypothetical synthetic agents themselves, one statement may be made with confidence. When reversible reactions, such as those investigated by Rosenthaler and by Beitzke and Neuberg, are in question, if a certain agent is found which accelerates the reaction in one direction but not in the opposite direction, such agent cannot be a catalyst and it does not therefore concern us here.

Do Enzymes form Constituents of the System in Equilibrium?

Facts of two kinds, which must be referred to in the present section, have led some writers on the theory of enzyme action to advocate the view that the above question should be answered in the affirmative.

In the first place, the equilibrium-position is usually different when brought about under the influence of an enzyme from what it is when brought about by an inorganic catalyst, such as acid. This fact has already been discussed briefly (p. 7 above), and the circumstance has been pointed out that a very small amount of energy, which might conceivably arise from the surface energy of a heterogeneous system, would be sufficient to account for the difference.

The work of Abel [1907, 1 and 1907, 2] on catalysis by formation of intermediate compounds between catalyst and substrate, indicates also the possibility that, in the case of enzyme action, a part of this compound might be left undecomposed at the end of the reaction. In this way, the catalyst would form part of the final chemical system in equilibrium brought about under the control of mass action. But it is to be remembered that the real active mass of an enzyme is extraordinarily small in comparison with the other components of the system, so that it does not seem that it can have any appreciable effect by mass action in affecting the position of equilibrium. The better the methods of purifying enzymes become, the more minute is the amount of enzyme required to produce a given result. The adsorption by enzyme, or by impurities associated with it, might have a very slight effect, but would be practically imperceptible.

In the second place, it has been found by various observers that the reaction appears to come to an end at different degrees of completion according to the amount of enzyme added. I may state at once that a very careful consideration of these results has led me to the conclusion that, in no case, is the proof given that equilibrium was actually reached.

The valuable and painstaking work of Tammann [1889, 1892,

1895] is often quoted in support of this view of a "false" equilibrium, or, otherwise put, of a quantitative relation between the amount of enzyme present and the total amount of change. Tammann himself, however, does not interpret his experiments in this way. The main object of his work was the investigation of the rate of spontaneous decay of the enzyme and the part played by the products of the reaction in accelerating this decay or in causing a paralysis of the activity of the enzyme. So far as I can find, the word "equilibrium" is not used at all in connection with the question. It is plain that, when the enzyme has disappeared from the sphere of action, no further effect beyond the very slow spontaneous course of the reaction, if present, is to be expected. The paralysis caused by accumulation of products, unless it has gone so far as to destroy the enzyme, can be annulled by removal of these products, or even by dilution. In such cases, the reaction may be made to recommence. It appears that the paralysis referred to here is not usually the result of a true equilibrium due to the opposing action of a reverse synthetic change, but to an injurious effect of certain chemical substances on the enzyme itself. In general, the recommencement of a reaction when the products are removed, may be the upsetting of a true chemical equilibrium by removal of a part of its components, but is more commonly brought about by removing substances which had caused stopping of the action of the enzyme before its work was completed.

It will be noticed that the cases in which this apparent attainment of a false equilibrium occurred were those in which very small amounts of enzyme were used. If the reader will refer back to figure 1 (p. 18), he will see that in the curve of action of the least amount of trypsin there is an indication of a result of this kind; it was not, however, very well marked, the continuation of the curve, for which the page is not sufficiently large, approached much nearer to the position of the other curves, as in fact its upward slope indicates. In the other curves, the position of equilibrium is identical; although, and this is an important point, the relative concentrations of enzyme were sufficiently different to produce very different rates of hydrolysis, so that the amount of enzyme present was in no case maximal.

Now, when we are dealing with very small amounts of enzyme, the difficulties of experimental certainty are great. There are several circumstances which contribute to this uncertainty. In the first place, the rate of change in the later stages, when the substrate concentration has diminished to a large extent, is, by mass action, very slow, even when the enzyme is present in full amount. Much more so, when the concentration of the enzyme is minimal, and in many of

Tammann's curves, it will be noticed that, although the upward slope of the curve is small, it has not entirely ceased to rise at the end of the figure. If more measurements had been made a week later, further change would undoubtedly have been detected in these cases. In the second place, Tammann himself records the fact, that in cases with very small amounts of enzyme, it was found, at the end of the experiment, that the enzyme had been destroyed. I can confirm this latter fact, and, in order to be certain of the absence of even a trace of emulsin, I concentrated the solution under reduced pressure and found that there was actually complete absence. In the third place, many of the reactions in question do actually proceed at a very slow, but measurable, rate without the presence of any enzyme. This fact adds further difficulty and is especially applicable to the experiments of Bradley [1910] on the action of lipase, in which the amount of triolein hydrolysed seemed to be in proportion to the actual amount of enzyme present. In some unpublished experiments with triacetin and the lipase in "pancreatin Rhenania," using three concentrations of enzyme, I noticed that, up to the fourth day of action, the rate of change was proportional to the concentration of the enzyme, so that the points reached by this time on the curves of hydrolysis varied in height. From this time onwards, the larger concentration of enzyme continued its curve, while the smaller concentrations had fallen to a very slow rate, which seemed to be the same in both. It appeared that, given time enough, all would ultimately have arrived at the same point, but the extremely slow rate made it impossible to form a conclusion as to whether this rate had any connection with the enzyme. The conclusion to be drawn is that, owing to these three causes of error, it is practically impossible to draw conclusions from very small concentrations of enzyme.

It is unnecessary to remark that no catalytic effect is to be expected after the enzyme has ceased to exist as an active component of the system. In such cases, addition of fresh enzyme causes renewed hydrolysis. It is somewhat more difficult to account for cases recorded in which addition of more substrate appears to have caused a renewed change in a system in "equilibrium". There are several possibilities. The equilibrium may have been a genuine one in a reversible reaction, although this is not probable in such cases as salicin and emulsin, or protein and trypsin. If the enzyme was still present in fairly large amount, the addition of more substrate would, by mass action, cause increased rate of hydrolysis. Even when the reaction was progressing so slowly, under the influence of minimal quantities of enzyme, that it appeared to have ceased, the addition of more substrate would increase

its rate to visibility. The effect of concentrating or diluting the whole system would depend on the actual concentration in substrate. If this were small, concentration would clearly increase the rate of a minimal change. Above a certain concentration, increase, as we shall see later, retards reaction, probably by mechanical action, so that dilution might increase a minimal rate in such a system. Further investigation is needed.

In connection with the effect of products of the reaction, it is interesting to note that, in Tammann's experiments, the injurious or paralysing action does not seem to depend on any particular chemical affinity of the enzyme to them, since, while some products, such as benzaldehyde, hydroquinone, and hydrocyanic acid are active, others, such as glucose, alcohol, and glycerol are comparatively inactive. In the case of benzaldehyde, actual precipitation of the enzyme occurred, and it seems probable that the other substances produce their effects by altering the colloidal state of the enzyme. Ether, which is not a product of the action of emulsin, has also a paralysing action.

The effect of *temperature on the equilibrium-point* is of some theoretical interest. In the case of glycerol-glucoside, where there was no doubt of the actual attainment of equilibrium, I could not detect any change by alteration of temperature and, indeed, since the reaction is practically thermo-neutral, van't Hoff's "Principle of Mobile Equilibrium" would lead us to expect that any change would be inappreciable. On the other hand, Tammann [1892, p. 285] found *less* hydrolysis of salicin in equilibrium at temperatures below 30° than at higher temperatures. Since this reaction is either thermo-neutral or very slightly exothermal (Herzog [1910, p. 203]), van't Hoff's principle implies no difference, or slightly *more* hydrolysis at the lower temperature. It seems most probable that, owing to the very slow action of the enzyme at the lower temperature, actual equilibrium had not been attained when the system was raised to the higher temperature. If the experiments were done at the same time on separate preparations, the enzyme might have ceased to exist in that one at the lower temperature before it had time to finish its work. The experiment should be made by first obtaining equilibrium at 46°, or thereabouts, and then cooling to the lower temperature, adding more enzyme, if necessary.

Under this heading there remains to be mentioned some work by Brailsford Robertson [1908, 2] on the action of strong pepsin solutions on the products of peptic digestion of caseinogen. A precipitate is produced which is regarded as "paranuclein". The amount formed is proportional to the amount of the pepsin solution added, and the results are stated to be evidence for a special synthesising form of

pepsin and for a quantitative relation between the enzyme and the total amount of change produced by it. On investigation of this reaction, I found [1913, pp. 261-63] that it is not an enzyme action at all, but a colloidal precipitation phenomenon. It is only produced by particular preparations of pepsin, and even these lose their activity in solution, if allowed to digest, although they are still strongly peptic. On the other hand, the enzyme can be destroyed by alkali, leaving the precipitating action unimpaired. The constituent of the digest of caseinogen which takes part in the precipitate appears to be nuclein, but what is the nature of the constituent of certain scale preparations of pepsin which is concerned I am unable to state, except that it is digested by the pepsin also present.

A word must be said with respect to the experiments of Gay and Brailsford Robertson [1912], who show that anaphylaxis is produced to the synthetic paranuclein by previous injection of paranuclein itself, but not by the peptic digest from which the synthetic product is prepared by the action of pepsin. The explanation of these results appears to me to be that the paranuclein, owing to the way in which it was prepared, which is, however, not stated, contained the substance, probably nuclein, which is also the active component of the colloidal complex precipitated by pepsin. If this is so, the same substance is injected in both cases. At all events, proof is wanting that this was not the case. The reason why the peptic digestion products gave no reaction is because they contain the "nuclein" in question in such extremely small quantity. It is necessary that paranuclein prepared by the method used in these experiments should be tested for precipitation by pepsin and also the substance precipitated by 0·2 per cent. hydrochloric acid from the peptic digest should be injected as antigen for anaphylaxis to synthetic paranuclein. I may point out that the substance in question is not precipitated by acetic acid, except in high concentrations.

Finally, it may be pointed out in reference to the supposed "false" equilibrium in which the enzyme takes part, that enzymes are insoluble in the ordinary sense of the word, so that they form a separate phase in the system and cannot, except so far as concerns their surfaces, take part in the chemical equilibrium as usually understood. The question of heterogeneous equilibrium is a very complex one, but has been investigated by Willard Gibbs [1878]. His work, however, can be recommended only to the expert mathematician. We shall find abundant evidence in the succeeding pages of the heterogeneous nature of enzyme systems.

Synthesis of Proteins.

Nothing has yet been said as to synthetic action on the part of proteoclastic enzymes. As pointed out by Leathes [1906, p. 132], the facts as to protein synthesis in the organism distinctly indicate a reversible enzyme action. The experiments of Loewi [1902], taken in conjunction with those of Henriques and Hansen [1904], have shown that animals are able to maintain their nitrogen-content on a diet in which the only nitrogenous bodies are the products of a prolonged tryptic and ereptic digestion ; but that the products of acid hydrolysis are unable to take their place. To quote the words of Leathes : "There appears to be some kind of linkage between certain groups in the protein molecules which is not uncoupled by the enzymes in the body, and that when it is uncoupled, as in acid hydrolysis, it is impossible for it to be coupled up again in the body. This combination, which the cells can neither take to pieces nor put together again, must be present, in order that the other component parts of the protein molecule may gather about it and group themselves round it when the synthesis of protein is to occur. These considerations appear to suggest that the synthetic processes here involved may be the work of the same agent as the hydrolytic, the limitations in its hydrolytic power determining the limitations of its synthetic activity, as in reversible zymolysis." A detailed investigation of the hydrolysis of proteins by pepsin, trypsin, and by acid will be found in the paper by Henriques and Gjaldbaek [1911, **2**].

More direct evidence of protein synthesis is not easy to get. The conditions are undoubtedly very complex, so that it is perhaps not to be expected that completely satisfactory results will be obtained until the necessary conditions are better understood.

A. E. Taylor [1907] has described the synthesis of a protamine by the action of trypsin, obtained from a mollusc, on the products of a tryptic digest of the same protamine. The supposed synthesis of paranuclein by pepsin has been referred to in the preceding section.

The bodies known as "plasteins" are, perhaps, to be looked upon as results of synthetic action. Danilewski [1886] showed that rennet preparations produced a precipitate in concentrated solutions of Witte's peptone, while subsequent workers found that the same effect was produced by pepsin and papain. These bodies are formed under such conditions that a reverse reaction would be expected to be most favoured ; the amount found was greater the higher the concentration of the peptone solution used. It does not appear, however, that these plasteins are necessarily the same as the original protein from which

the peptone was made: the plastein from caseinogen peptone does not, on acid hydrolysis, give quantitatively the same result in amino-acids as the caseinogen itself. This is not surprising, when we consider the variety of possible hydrolytic products.

Henriques and Gjaldbaek [1911, I, p. 517], as the result of a detailed investigation of the action of pepsin on concentrated solutions of peptone, come to the conclusion that a synthetic process goes on, which can be followed by Sörensen's formaldehyde titration method. The more strongly hydrolysed the substrate is initially, the less complex is the structure of the synthetic substance, although it is produced in larger quantities; this fact is especially noticeable in the case of the products of acid hydrolysis of casinogen. In some cases, it contains very few more free NH_2 groups than genuine proteins. The precipitation of a complex colloid by the action of some constituent of particular pepsin preparations on the products of caseinogen digestion, described in the preceding section, shows that care is required in the interpretation of similar results. In fact, I found that Witte's peptone gave the reaction. The authors, however, believe that they have excluded errors of this kind, and the fact that the formaldehyde titrations of the whole system change in the direction of synthesis appears to be good evidence. Probably the circumstance that the synthetic product is thrown out of the system by precipitation enables the reaction to proceed sufficiently far to be capable of detection.

A concentrated solution of products of tryptic digestion of caseinogen also shows a diminution of electrical conductivity under the action of trypsin, which seems to indicate a synthetic process.

Experiments made by Abderhalden and Rona [1906, p. 35] to detect whether there was any synthesis of polypeptides from their constituent amino-acids under the influence of tissue enzymes led to no result.

Although the direct evidence on the subject of protein-synthesis is at present meagre, the phenomena seen in trypsin digests are quite what would be expected if equilibrium in a reversible reaction be the explanation of what takes place. Such phenomena are (1) retardation due to accumulation of the products of the reaction, (2) recommencement of a reaction which had apparently come to an end, if the products be removed by dialysis, or other means, or if their concentration be reduced by dilution. In the case of this enzyme, however, the products act also in another way in diminishing the rate of change, namely, by reducing the alkalinity of the solution, as will be shown in a later chapter.

Synthesis by Amino-Acids.

The important synthesis by the catalytic agency of amino-acids described by Dakin [1909] has been already referred to. If this reaction is a reversible one, it would be of considerable interest to know whether hydrolysis of the product is also accelerated by the same catalyst that synthesised it. I find that the cinnamylidene-malonic acid produced from cinnamic aldehyde and malonic acid by the catalytic action of glycocoll, in concentrated solutions of the constituents, is an unstable body, readily oxidised. This synthesis is accompanied by a fall in electrical conductivity, as would be expected. Dilute solutions of the synthetic acid undergo a slow increase in conductivity, which is accelerated by the addition of the catalyst glycocoll. Too much stress must not be laid on this fact until we know more of the chemistry of the process.

Asymmetric Synthesis.

Rosenthaler [1908] has described an interesting case of an asymmetric synthesis by emulsin. When a mixture of hydrocyanic acid and benzaldehyde is acted upon by emulsin, under certain conditions, an optically active benzaldehyde-cyanhydrin is formed. As to the mechanism of this reaction see Note E.

Armstrong and Horton [1910] state that they have been able to confirm this result of Rosenthaler's. I have myself been unable to do so. Although I tried two different preparations of emulsin and followed closely the instructions of the author, I could obtain no formation of an optically active body, nor did my enzymes cause any asymmetric hydrolysis of the racemic benzaldehyde-cyanhydrin. Considerable symmetrical hydrolysis occurred and amygdalin was rapidly attacked.

It is interesting that Bredig and Fiske [1912] have found a similar asymmetric synthesis to be brought about by the agency of optically active bases as catalysts. Taking quinine, dextro-rotatory, a dextro-rotatory cyanhydrin was obtained. The lævo-rotatory base, quinidine, on the other hand, caused the production of lævo-rotatory cyanhydrin.

It should perhaps be mentioned, in reference to Rosenthaler's results, that Bredig and Fiske were unable to obtain concordant results by using the method of the former observer for estimation of the active cyanhydrin and that objection to the method had been already made by Wirth [1911]. On this account, although Rosenthaler's synthesis is not improbable, and has been confirmed by Armstrong, caution must be exercised in accepting the details of his work.

Most of the cases of synthesis by enzymes, described in the present chapter, are, of course, asymmetric, with the exception of those by lipase.

CHAPTER VI.

THE VELOCITY OF REACTION AND THE VARIOUS CONDITIONS AFFECTING IT.

THE function of enzymes, as catalysts, being to change the rate of reactions, it follows that the study of their action, apart from that of the nature of the products formed, consists essentially in the investigation of the velocity of reactions and the factors which have an influence upon this.

Use of Mathematics in Biology.

In the discussion of this problem a certain amount of use must be made of mathematical forms of expression. Since there is a tendency to decry the introduction of formulæ into biological science, a few words are advisable upon the value of such a mode of treatment.

Although it may be perfectly true that by mathematical analysis no new facts are discovered, it is none the less true that the expression of experimental results in a formula shows their relation to known laws in a way which is otherwise very difficult or impossible to attain. Further, as Arrhenius [1907, p. 7] points out, such a procedure enables one to see whether all the factors have been taken into account; instances of this will appear in the course of the present chapter. Even an empirical formula may assist in deciding whether irregularities are due merely to experimental error or otherwise.

The comparison by Huxley of mathematics to a mill, which cannot make fine flour out of peas and only turns out in another form what is put into it, is frequently quoted. But the fact that the material turned out is in a much more useful form than when it was put in is sometimes forgotten.

Mass-Action.

The law of mass-action tells us that a reaction proceeds at a rate proportional to the concentration of the reacting molecules. The number of times per unit of time that one molecule encounters another, with which it can enter into reaction, is obviously related to the number present in a given volume, that is, to the concentration. So that when the reaction can be expressed as the change in concentration of one kind of molecules, the rate of change at any moment is proportional to the amount of this substance still left undecomposed. Such a case is the hydrolysis of saccharose under the action of hydrion in dilute solution. It is true, of course, that for each molecule of sugar inverted a molecule of water is taken up, but as the reaction is taking place in excess of water this factor is not appreciable. If we call x the amount of sugar inverted in the time t, the average rate of the change during the time t is $\frac{x}{t}$, and if C is the concentration of saccharose at this time, the velocity is proportional to it, *i.e.*,

$$\frac{x}{t} = k\mathrm{C}$$

k being some constant.

But, owing to the continuous hydrolysis of the sugar, its concentration (C) is not the same at any two consecutive periods of time, so that the above equation is only correct when t is so short that no appreciable change has taken place in the sugar-content. This is expressed in the notation of the differential calculus thus:—

$$\frac{dx}{dt} = k\mathrm{C}$$

or, since x is proportional to C,

$$-\frac{d\mathrm{C}}{dt} = k\mathrm{C}$$

the minus sign indicating that C (= concentration of saccharose) is diminishing. The symbols dx and dt are to be taken as wholes, and simply mean that x and t are to be taken so small that the velocity has not changed during the time t.

Uni-molecular Reaction.

A reaction such as this, which can be adequately treated as consisting of the change of concentration of one substance, is called a "unimolecular" reaction and is expressed by the equation given.

Now it is clear that to make any practical use of the equation some means must be devised in order to render it applicable to data in which the time is sufficiently long to be measured; such a process is known as "integration". It is impossible in the limits of this book to describe the method in detail, and the reader is referred to the Introduction to Mellor's *Chemical Statics and Dynamics* for further information. Suffice it to say that the process is an artifice by which an exceedingly large number of exceedingly small quantities are added together, so that, *e.g.*, all the values of $\frac{dx}{dt}$ during the space of ten minutes are added together. The change of concentration in such times can be determined by some one of the methods previously described.

Newton's Law of Velocities.

It is interesting to remember that any process which tends towards an equilibrium becomes slower and slower as the final state is more nearly reached. One may regard the driving force as becoming less and less. Such cases are the equalisation of temperature between neighbouring hot and cold bodies, the flow of water from a full cylinder to an empty one, when the cylinders are connected by a tube at the bottoms, as well as the reversible chemical reactions with which we have to deal in this chapter. The law is sometimes spoken of as *Newton's Law of Velocities.*

Logarithmic Curve.

If it be called to mind that the kind of process with which we have to deal is one where the velocity at any given moment depends on that of the moment preceding, and that such a process when plotted out as a curve forms a logarithmic curve, that is a curve such that one set of co-ordinates is a series of numbers and the other set the logarithms of these numbers, it may help us to understand why the integral of our differential equation has a logarithmic form. This integral may be put in various forms, but for practical use the following form is the most appropriate one for enzyme work, in which the initial and end points are apt to be uncertain:—

$$k = \frac{1}{t_2 - t_1} \log \text{nat} \frac{C_1}{C_2}$$

where k is the velocity-constant of the original equation and C_1 and C_2 the concentrations of the substrate at the times t_1 and t_2 respectively, reckoned from the commencement of the reaction. As will be seen, any two determinations during the course of the reaction can be used for the calculation of the value of k.

Another form, from which, in fact, the above is derived and which is often useful, is

$$k = \frac{1}{t} \log \text{nat} \frac{a}{a - x}$$

in which t is the time which has elapsed since the beginning of the reaction, a is the initial concentration of the substrate and x is the amount of products formed during the time t, so that $a - x$ is the substrate-concentration at the end of the time t.

Inversion by Acid and by Enzyme.

The following table will serve to show the kind of values obtained in a uni-molecular reaction, *viz.*, the inversion of cane-sugar by acid; it will be seen that the velocity-constant, as calculated by the above equation, is practically the same throughout, within the limits of experimental error:—

Time in minutes.	Rotation.	Velocity-constant.
0	46·75°	0·001330
30	41·00°	1332
60	35·75°	1352
90	30·75°	1379
120	26·00°	1321
150	22·00°	1371
210	15·00°	1465
330	2·75°	1463
510	– 7·00°	1386
630	– 10·00°	
∞	– 18·75°	

Now suppose that, instead of using acid, the enzyme invertase had been employed and the velocity-constant calculated by the same formula, we obtain the following results (V. Henri [1903, p. 55]):—

Time in minutes.	Proportion inverted $\left(\frac{x}{a}\right)$.	Velocity-constant.
66	0·084	0·00058
168	0·220	64
334	0·426	72
488	0·581	77
696	0·746	85
1356	0·952	97

The constant shows a steady *rise*.

Take now a corresponding series of values from the experiments of Frankland Armstrong [1904, **I**, p. 506] on the hydrolysis of milk-sugar by lactase:—

Time in hours.	Velocity-constant.
1	0·0640
2	0·0543
3	0·0460
5	0·0310
24	0·0129

In this case, unlike that of invertase, there is a steady *fall* in the values of the velocity-constant.

Another case is that of trypsin, the following table being taken from an experiment of my own :—

1st	10 minutes	k = 0·0079
2nd	,,	0·0046
3rd	,,	0·0032
4th	,,	0·0022
5th	,,	0·0016
7th	,,	0·0009
9th	,,	0·0007

Here, again, there is a marked diminution in the values of k as calculated by the simple uni-molecular formula.

Causes of Divergence from Simple Law.

What is the explanation of these disagreements with the said law?

In the first place, it must be noted that cases in which the velocity is greater at any given time than that calculated by the uni-molecular formula are unusual ; we will therefore consider first the opposite case, which is that found to apply to most enzymes.

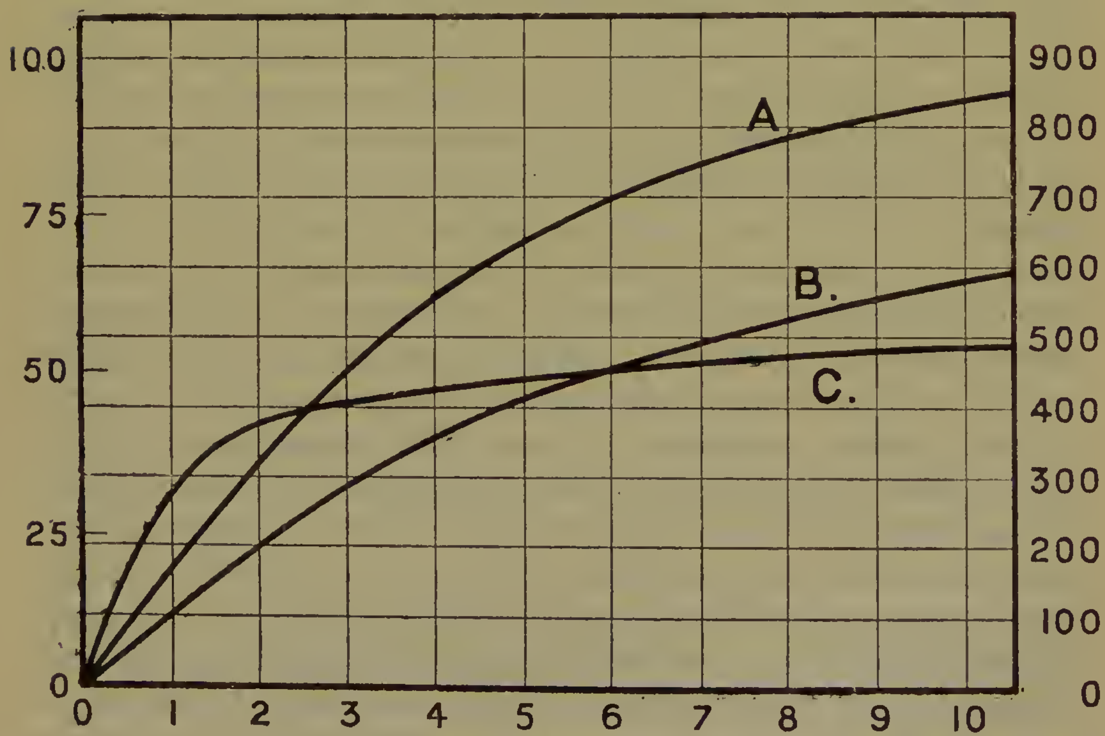


FIG. 2.

The contrast between the three cases dealt with will be made clearer if they are put in the form of curves, as is done in Fig. 2.

In this figure curve A is that of the action of invertase, taken from one of Victor Henri's experiments; the ordinates are given by the numbers on the left of the figure and represent proportions per cent. inverted; curve B is the true logarithmic one of acid inversion, and curve C is that of the changes in electrical conductivity in the action of trypsin on caseinogen, taken from one of my own experiments, the ordinates being given in gemmhos (= reciprocal megohms) on the right of the figure. The numbers on the axis of abscissæ denote hours and apply to all the curves.

1. *Disappearance of Enzyme.*

It is found by experiment that in many cases the enzyme itself disappears in the course of its action. Since the amount of the catalysis is always in direct proportion to the concentration of the catalyst, although, as we shall see, not always in linear proportion, a gradual destruction of the enzyme would lead to a slowing of the reaction greater than that due to the diminished concentration of the substrate. We have already seen that in fairly pure solution most enzymes are unstable, especially at a temperature of 38°, but that, on the other hand, in the presence of their substrates or products they are much more stable. Trypsin is one of the most unstable of enzymes when these protecting influences are not present; Vernon [1902, p. 378] found that 70 per cent. was destroyed in 0·4 per cent. sodium carbonate solution in an hour. However, in an actual digestion mixture I found, in a series of experiments made in the manner given by V. Henri [1904, pp. 302-303], that no diminution of the activity of the enzyme could be detected up to six hours in the conditions of the experiment from which the data of the table above were obtained, and very little up to seven hours. The method was as follows: in order to have all the other conditions, with exception of the enzyme, constant, at various stages in the course of a series of determinations of the electrical conductivity of a reacting mixture of caseinogen and a known initial concentration of trypsin, samples were removed and immediately immersed in small flasks in boiling water, in order to destroy the enzyme. To each of these trypsin was then added in the amount requisite to make the concentration the same as it was at the beginning; the rate of change was now compared with that of the original solution at the same stage. If the samples with fresh enzyme showed a greater rate of change than the main digest at the same stage, it is obvious that there was less enzyme present in the latter than had been originally put in, since all other conditions were identical. The results showed that the destruction of enzyme was not the chief cause of the falling off of activity, since there was no appreciable destruction up to eight hours. Similar results were obtained by Tammann in the case of emulsin.

2. *Effect of Products.*

There is then some other cause for the deviation from the logarithmic law. This is found, by experiment, to consist in the effect of the products of the reaction. When these are added to a reacting mixture, a change in the velocity of the reaction occurs such as shows itself when the same products make their appearance during the normal course of the reaction.

This effect of the products appears, in the case of trypsin, to be due mainly to reduction of OH' ion concentration, since Walters [1912] finds that, in dilute solutions, the effect of the products, as such, is comparatively slight.

3. *Combination of Enzyme with Substrate.*

There are several ways in which this effect of the products of the reaction shows itself. In the first place, there is abundant evidence that a combination of some kind is formed between the enzyme and the substrate preparatory to the action of the former. There is also a similar combination between the enzyme and products, as would naturally be expected, if both hydrolytic and synthetic processes are catalysed; if combination between enzyme and substrate is requisite for the former, then presumably combination between enzyme and products is requisite for the latter. But let us see what evidence there is of such combinations of either kind.

As long ago as 1806 Clément and Désormes [1806] put forward one of the first explanations of a catalytic process in their theory of the action of nitrous acid in the oxidation of sulphur dioxide to sulphuric acid. This explanation consisted in the formation of a temporary combination between the substrate and catalyst in the shape of what is now called nitrosulphonic acid, which afterwards decomposed with formation of sulphuric acid and regeneration of the catalyst. The actual proof of such intermediate combinations has only been given in more recent times by Brode [1901], as already mentioned. A similar course of events is also now regarded as the most acceptable theory of the action of enzymes.

The enzyme appears as a rule to enter into a state of association with some particular molecular grouping in the substrate, so that, if this is an uncommon grouping, the enzyme will be very "specific"; thus invertase enters into relation only with fructose, maltase only with bodies having the structure of the α-glucosides and so on. Such an intimate correlation is particularly well seen in the case of the various yeast-enzymes acting on disaccharides, which were investigated by Emil

Fischer, and which led him to formulate his famous simile of the "lock-and-key" relationship [1894, p. 2992]. This implies a very close similarity in configuration between enzyme and substrate, and since, as we have seen, it applies also to optical opposites, it makes it probable that the enzymes in question are themselves optically active. There is, moreover, evidence that other enzymes are optically active. The work of Dakin [1904] on hydrolysis of optically active esters by the lipase of liver affords evidence on this point as well as on the general question of combination. It was found that, when the optically inactive mixture of the two esters of mandelic acid was acted on by this enzyme, the dextro-component was hydrolysed more rapidly than the lævo-component. In this way it happened that the percentage of dextro-mandelic acid in the products became greater at first than that of the lævo-acid, so that the mixture was optically active; as the reaction proceeded the relative amounts approximated more and more, until finally both were present in equal quantity and the mixture became again optically inactive. These facts can only be satisfactorily explained on the hypothesis that the enzyme itself is optically active and forms addition compounds with the esters. As Dakin puts the matter: "The dextro- and lævo-components of the inactive ester first combine with the enzyme, but the latter is assumed to be an optically active asymmetric substance, so that the rates of combination of the enzyme with the d- and l-esters are different. The second stage in the reaction consists in the hydrolysis of the complex molecules of (enzyme + ester). Since the complex molecule (enzyme + d-ester) would not be the optical opposite of (enzyme + l-ester), the rate of change in the two cases would again be different. Judging by analogy with other reactions one might anticipate that the complex molecule which is formed with the greater velocity would be more rapidly decomposed. In the present case it would appear that the dextro-component of the inactive mandelic ester combines more readily with the enzyme than the lævo-component does, and that the complex molecules (d-ester + enzyme) are hydrolysed more rapidly than (l-ester + enzyme), so that if the hydrolysis be incomplete dextro-acid is found in solution and the residual ester is lævo-rotatory." I would, in passing, call attention to the fact that in these experiments, unlike the case of emulsin acting on the methyl glucosides, the lipase does not show itself to be capable of acting on one of the two optical isomers only; it hydrolyses both but at unequal rates. This circumstance, on the face of it, looks more like a kind of combination approximating to a physical type rather than to chemical union in the strict sense.[1]

[1] See Note D.

That there is a marked "affinity" for certain optically active groups is shown by the work of Fischer and his coadjutors [1905], Abderhalden and others, on the relation of trypsin to di- and polypeptides. Without entering into details, it must suffice to say here that it is impossible at present to give any general rule as to which of these compounds is hydrolysed by trypsin; only compounds of naturally occurring amino-acids are attacked, and amongst these there is a preference shown for those containing tyrosine or leucine and, in a somewhat less degree, for those containing alanine. At the same time, many curious preferences are to be observed, for example, alanyl-glycine is hydrolysed, but glycyl-alanine is not, l-leucyl-l-leucine is attacked, but neither l-leucyl-d-leucine nor d-leucyl-l-leucine is, and so on. For further details as to this problem, Part II. of the monograph by Dr. Aders Plimmer in this series must be consulted.

Further evidence upon the combination of enzyme and substrate was afforded by the observation of O'Sullivan and Tompson [1890]. They found that invertase will withstand uninjured a temperature 25° higher in the presence of cane-sugar than in its absence. As they point out, it is difficult to see how this could happen unless the enzyme entered into some kind of union with the sugar.

Ratio of Velocity to Concentration of Enzyme.

Another phenomenon, which is impossible to explain except on the hypothesis of a combination of this kind, is the law of the reaction-velocity in the initial stages of certain enzyme actions when low concentrations of the enzyme are used. Duclaux [1883, 1899] found that, under these conditions, the rate of change, in the case of invertase, did not follow the law of mass-action, but that the amount of cane-sugar inverted was directly proportional to the time of action, or in other words, that the same quantity was hydrolysed in the second ten minutes as that hydrolysed in the first ten minutes: the curve instead of being logarithmic, became a straight line. Adrian Brown [1902] showed that, when cane-sugar solutions of varying concentrations were hydrolysed under the influence of invertase, in the early stages of the reaction the amount inverted in equal times was nearly the same in all. According to the law of mass-action these amounts should have been proportional to the concentrations of the substrate. In order to explain this result, Adrian Brown assumed that not only is there formed a compound of enzyme and sugar, but that this exists for an appreciable time; consequently a definite quantity of the enzyme can only effect a limited number of complete molecular changes

in a given time; whatever the available mass of the substrate may be, if it is greater than the amount of enzyme which is present and with which it can enter into combination, no increase in the amount changed is possible. If the ratio of the enzyme to the cane-sugar be greater than a certain value, the amount of the latter hydrolysed is directly proportional to the concentration, as the logarithmic law requires.

This question is further discussed in a paper by Horace Brown and Glendinning [1902] on the relations of starch and amylase. They point out that, when the concentration of the enzyme is very small relatively to that of the starch, in the early stages of the reaction, as long as this excess of substrate remains unhydrolysed, the amount of starch per unit volume will be very large compared with the amount of the combination of starch and enzyme. So long, therefore, as the concentration of the unchanged substrate remains very large in relation to that of the combination, the latter will remain nearly constant in amount and equal amounts of starch will be hydrolysed in equal times, the curve being a straight line. Subsequently, when the concentration of the starch has been much reduced, the amount of the combination and consequently the hydrolysis of the sugar will follow more closely the law of mass-action. It is pointed out also that this explanation is in agreement with experimental facts.

Similar results were obtained by Frankland Armstrong [1904, **1**, p. 508] in his work on lactase, maltase, and emulsin. The following numbers give the amounts of milk-sugar hydrolysed in forty-six hours by a very small amount of lactase acting on different strengths of the solution of sugar:—

Solutions containing	Proportion Hydrolysed.	Actual Weight.
10 per cent.	22·2	2·22
20 ,,	10·9	2·18
30 ,,	7·7	2·21

Experiments in which the proportion of enzyme present was large relatively to the concentration of the sugar gave a different result:—

Milk-sugar per 100 c.c.	Amount Changed in Three Hours.	Velocity-constant.
1·0 gramme	0·185	0·0296
0·5 ,,	0·098	0·0298
0·2 ,,	0·0416	0·0337

The amount hydrolysed was in exact ratio to the concentration of the sugar, while the velocity-constant was nearly the same in all.

Combination between Enzyme and Products.

When we come to examine the evidence for combination between enzyme and products it is found that it is of a similar nature to that just dealt with, but perhaps less direct.

O'Sullivan and Tompson [1890], in the work above mentioned, showed that invertase was protected from the action of heat by products of the inversion of cane-sugar, as well as by the sugar itself. Trypsin is much more stable in the presence of either substrate or products than alone. This was shown for peptone by Starling and myself [1903], and for amino-acids by Vernon [1904, p. 354].

There is no doubt that, in the case of the sucroclastic enzymes, as investigated by Frankland Armstrong [1904, **2**, p. 520], there is a special retarding influence exerted by the respective products of the enzymes, lactase, emulsin, maltase, and invertase, on the rates of hydrolysis by these enzymes, an effect which is not shown to the same degree by other sugars. For example, fructose retards invertase, but has less effect on any one of the other enzymes, galactose has very little effect on maltase, but considerable retarding action on lactase.

At the same time, it is to be remembered that this influence is by no means so specific as thought at one time. Indeed it seems doubtful whether it has any connection with chemical relationships between enzyme and substrate. Philoche, for example [1908, p. 243], shows that fructose retards the action of maltase considerably more than glucose does. If there is chemical relationship between the enzyme and substrate, emulsin, which acts on a great variety of glucosides, whose only common constituent is glucose, should be especially retarded by this sugar, while the other constituent should be comparatively inert. The contrary is the fact. Glucose, indeed, appears to be a somewhat indifferent substance, so far as concerns its effect on enzymes. Of course, in concentrated solution, as a constituent of the system in equilibrium, it must, by mass-action, cause retardation of the hydrolytic component of the reaction.

In some cases, certain substances, which make their appearance as products of the reaction, benzaldehyde, for example, have been found to cause precipitation, or even destruction, of the enzyme. This fact goes far to suggest that many of the apparently specific retarding effects may be due merely to changes in the colloidal state of the enzyme, so that its active surface is decreased.

Rôle of Reversibility.

If we examine the data given by Frankland Armstrong we notice that, while both of the products have an action of the kind in question, the effect of one is usually more marked than that of the other. This was believed to be due to the fact that the enzyme in each case has the property of combining in a special manner with a particular sugar, and by this means is withdrawn from the sphere of action. From the consideration of relations of this kind, it is suggested that these sugar-splitting enzymes enter into relation with their substrates along nearly the whole of the molecule, but that a small degree of misfit, so to speak, prevents actual hydrolysis. To take the cases of the two methyl-glucosides and their relations to maltase and emulsin respectively, the state of affairs may be represented by the diagrams below (Fig. 3), in which it will be seen that emulsin, for example, is only "out of harmony" with the a-glucoside at the extreme top of the figure, yet, as we know, this is sufficient to prevent its action. It must be understood that these figures, being only in one plane, cannot represent the real shape of the molecules, so that they must be taken merely as a kind of shorthand to express the experimental facts.

Results of a similar nature were obtained by Abderhalden and Gigon [1907] in the case of the action of yeast press-juice on glycyl-l-tyrosine. The addition of the amino-acids d-alanine, d-valine, l-leucine, l-tyrosine, tryptophane and d-glutamic acid, which are constituents of polypeptides hydrolysed by the enzyme, were found to retard the reaction; but l-alanine and d-leucine have no effect. In other words, amino-acids, which are found in bodies upon which the enzyme acts, are able to enter into a combination of such a kind with the enzyme that this is withdrawn from the reacting system.

Tammann [1889] showed that the action of emulsin on amygdalin was retarded by any of the products of reaction, and I have myself recently found that the action of the same enzyme on arbutin (hydroquinone-glucoside) is retarded by both glucose and by hydroquinone and, indeed, to very nearly the same degree by both. A 0.2N solution of arbutin was hydrolysed by emulsin at 37·5° to the extent of 26·6 per cent. in twenty-five hours; a similar solution to which hydroquinone had been added to an amount such as to make the solution 0·1 N, showed a change of only 15 per cent.; another similar one, with glucose in place of hydroquinone, showed 13·5 per cent. hydrolysis, while a fourth which was 0·05N in both glucose and hydroquinone was hydrolysed to 13·25 per cent. Considering the great number of different glucosides which are attacked by emulsin it is very difficult to believe that the enzyme is able to enter into relation with the non-sugar part of all these bodies

which have such varied chemical constitution. If the phenomena can be explained in another way it is obviously to be preferred.[1]

So far as we know, all the reactions catalysed by enzymes are reversible, differing only as to the position of equilibrium; in some instances, as that of invertase, the equilibrium-point is so near that of complete hydrolysis that it needs careful investigation to detect that the latter is not quite complete. To return for a moment to the typical case of acid, alcohol, and ester, represented by the equation:—

$$\text{ester} + \text{water} \rightleftarrows \text{acid} + \text{alcohol},$$

let us suppose that we start with ester and water alone, with a lipase as a catalyst. At the commencement the reaction of hydrolysis will be rapid, while the reverse reaction of ester formation will be zero; as soon, however, as any perceptible amount of acid and alcohol are formed, the synthetic reaction will begin, at first very slowly

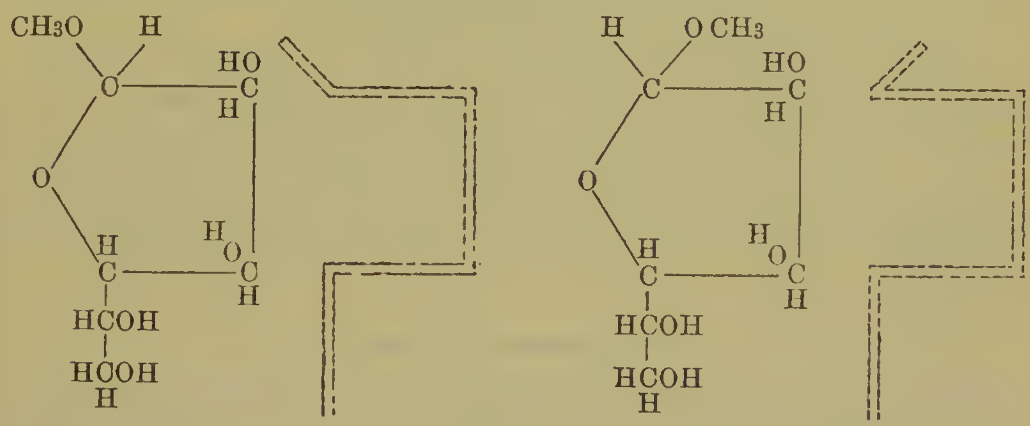


FIG. 3.

but gradually gaining in rate as the concentration of the hydrolytic products increases, while the hydrolytic reaction will become slower as the concentration of the substrate diminishes, until finally the rates of the two opposite reactions are equal and equilibrium is established. Now if we direct our attention to the hydrolytic process alone, we see that it is more and more counteracted by the opposite process as the reaction proceeds, so that the actual values obtained in an experiment are really the differences between two opposite reactions. As Benjamin Moore [1906, p. 79] rightly points out, this reverse reaction will show itself as a slowing of the hydrolytic process to a greater and greater degree as equilibrium is approached, even in those cases where the hydrolysis is practically complete.

The actual velocity of reaction at any moment is then to be regarded as the difference between two opposite processes. We see also why each of these processes can be accelerated by the addition of substrate or products respectively.

[1] For further considerations as to the "specificity" of enzymes the reader is referred to Note E.

Equilibrium a True One.

It will be remembered that Croft Hill showed that the equilibrium in the case of maltase was a genuine one, inasmuch as the same point was reached starting from either end, glucose or the disaccharide. The same fact has been shown by Bourquelot and Bridel [1913, pp. 68-72] in the case of β-ethyl-glucoside and emulsin, by Jalander in that of the lipase of the castor oil bean [1911, p. 475] and by myself [1913, pp. 240-43] in the case of β-glycerol-glucoside. Visser [1905, p. 276] believes that he has shown that this is also the case with salicin and emulsin, although the equilibrium-point is so near to that of complete hydrolysis that certainty is difficult to attain. However, the value obtained by Bourquelot and Bridel [1913, p. 14] agrees with that of Visser. This latter observer also found a slight synthesis of cane-sugar by means of invertase [1905, p. 275].

Robertson, Irvine, and Dobson [1909] also found that preparations of the enzymes in the leaf and stem of the sugar-beet caused the production of sucrose to the extent of 6 per cent. when allowed to act on a concentrated solution of invert-sugar. It is of interest that these observers found no invertase in the root itself during storage of sugar.

"Intensity-Factor."

When the velocity-constants for the hydrolytic process in the two cases are calculated from a formula which takes account of the two opposite reactions, it is found that these constants, just as when the reverse reaction is disregarded, still show a considerable amount of the same regular increase or decrease of the values of the velocity-constants during the progress of the reaction. It is evident, therefore that some other cause is also present. Visser [1905, p. 283] introduces the conception of "intensity" of action of the enzyme, and, by introducing appropriate factors in the formula, it was found that satisfactory regularity of the velocity-constants was obtained, even when the reverse reaction was neglected altogether [1905, p. 296]. We shall see presently what meaning is to be attached to this "intensity factor". It is perhaps not surprising that the reverse reaction has comparatively little influence on the rate of hydrolysis in the two instances dealt with; since the equilibrium-position is so near to that of complete hydrolysis, the velocity of the synthetic reaction must be very small; some approximate numerical values have been given in Chapter V.

Equilibrium in the Lipase System.

The important researches of Dietz [1907] were concerned with the action of pancreatic lipase. In order to avoid complications due to reactions taking place in steps, only univalent alcohols and monobasic acids, with the corresponding esters, were used. All the chief experiments were done with isoamyl alcohol and normal butyric acid. Since the alcohol and water were always in considerable excess, usually somewhere near five molecules of water to eight molecules of alcohol, both the hydrolysis and synthesis could be treated as unimolecular reactions and the calculations thus simplified. In order also to obtain the exact equilibrium the experiments were nearly always carried out from both sides simultaneously, on the one side a solution of ester in water + amyl alcohol was taken and on the other side an equally concentrated solution of butyric acid in amyl alcohol + water.

The first thing to notice is that the equilibrium-position, as shown by the final concentration of acid, is the same whether approached from the side of ester or from that of acid [1907, pp. 302-6]. It was found, moreover, that when enzyme preparations of different activity, as shown by the velocity-constants, were taken, the equilibrium was the same in all. Similarly, different amounts of the same enzyme preparation were without effect on this point. It may be remarked here that similar results were obtained by Visser with invertase and emulsin.

When experiments were made with different initial concentrations of the substrates, results were obtained which differed from what the law of mass-action demands. The probable reason of this will be given in the next chapter of the present work.

As regards the velocity-constants themselves, it was found that when the water-concentration of the butyric acid solution was low, the ester formation followed the logarithmic law of a simple reaction of the first order as would be expected; the rate of the reverse reaction, the ester hydrolysis, was therefore negligible.

On proceeding to higher concentrations of water, it is seen that, if butyric acid is the starting-point, a considerable part of it is not esterified, while, if the ester is the starting-point, it is partially hydrolysed. In this way the two velocity-constants can be measured. When this is done by the regular unimolecular formula, constant values in each case are obtained, within the limits of experimental error. The table below is a copy of one of those given by Dietz [1907, p. 305]; t = time in hours, T = millimols of acid per litre required to neutralise, k_1 = velocity-constant of the synthetic process, and k_2 = that of the hydrolytic process. The reactions took place in amyl alcohol containing 8 per cent. of water.

Ester Formation.		
t.	T.	k_1.
0·00	197·70	—
1·58	187·40	0·015
4·00	177·60	0·012
7·00	160·80	0·013
10·40	147·00	0·013
15·05	126·50	0·014
24·48	98·51	0·014
31·62	88·06	0·014
96·30	48·51	———
∞	45·90	0·014 in the mean.
Ester Hydrolysis.		
t.	T.	k_2.
0·00	0·00	—
2·95	6·86	0·0055
7·20	13·44	0·0049
16·40	24·25	0·0046
23·65	30·23	0·0045
45·07	40·30	0·0047
88·83	44·40	———
∞	45·90	0·0048 in the mean.

Fig. 4 will serve to give a general idea of the course of the change in these experiments. The ordinates represent the concentration of

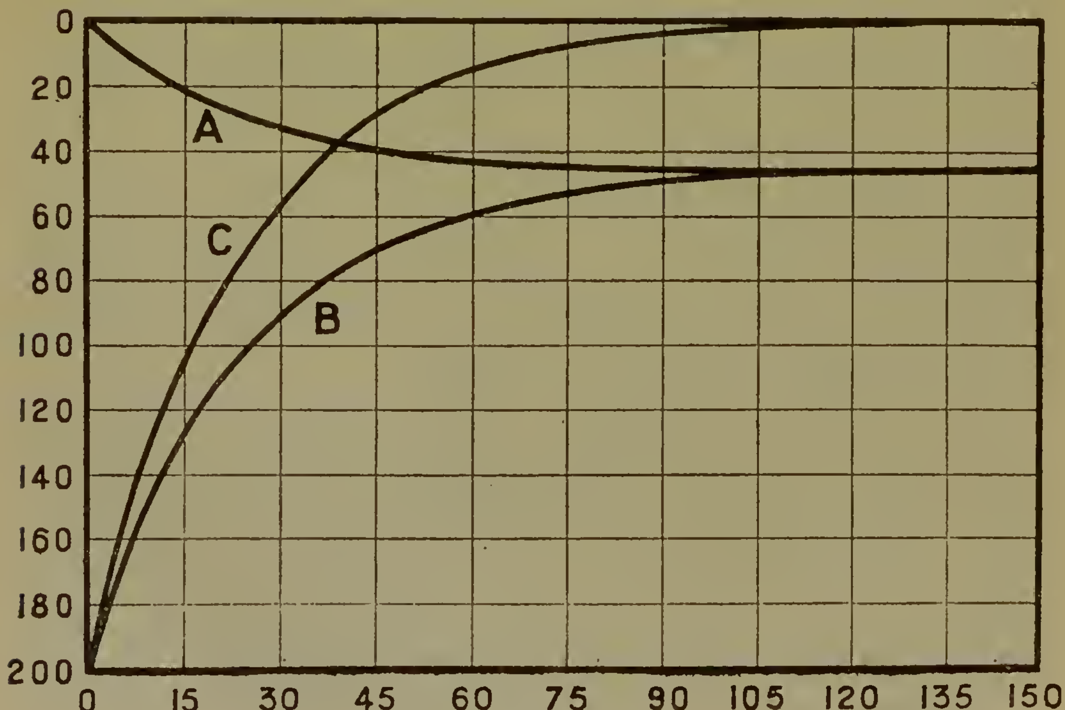


FIG. 4.

butyric acid and the abscissæ time in hours, so that the upper curve A is that of ester hydrolysis and the lower curve B that of ester for-

mation. Curve C is that which would be given if the reaction went to completion in one direction.

It will be remembered that the equilibrium condition is definable in two ways, either as the ratio of the concentrations of the bodies taking part in it, in the present case ester and acid, or as the ratio of the two opposite velocity-constants. From the data given in the tables above it is possible to obtain values in both ways. Calculated from the relative concentrations, it works out to be 3·3 and from the velocity-constants 3·4, a very satisfactory agreement. It is to be noted, however, that such good agreement was not always found.

A rather considerable time has been spent over the results of Dietz, since they form an analysis of a fairly simple case and will serve as a foundation on which further complications may be added.

A similar curve to that of Fig. 4, but relating to the glycerol-glucose-emulsin system, will be found in my paper [1913, p. 242].

Activity of Enzyme.

One of these complications is the factor called by Visser the "intensity" of the enzyme, which plays an important part in many reactions, but appears in the case of lipase not to affect the form of the expression for the reaction velocity. The simplest way in which this factor can be altered is plainly by variations in the concentration of the enzyme added. As we have seen, the rate of action is proportional to this concentration, so that if, during the progress of a reaction, anything happened which lessened the effective concentration of the enzyme, the reaction would be slowed. The combination of enzyme with its products is, in fact, one such circumstance, as it acts by removing enzyme from the sphere of action. But the activity of the enzyme may be affected in other ways without being actually removed. Trypsin, for example, is extraordinarily sensitive to the presence of alkali (hydroxidion); it is practically inert in acid or neutral solution, but is greatly assisted by the presence of alkali, and up to certain limits in direct ratio to the concentration of this latter as the following numbers show. A series of solutions containing 2·5 per cent. of caseinogen and the concentrations of ammonia shown in the first column were acted on by trypsin for one hour; the rises of conductivity given in the other column were found.[1]

Concentration of Ammonia.	Change.
0·05 normal	1770 gemmhos
0·03 ,,	1365 ,,
0·01 ,,	680 ,,

[1] For further details as to the influence of the reaction of the medium on the activity of enzymes, see the careful investigation of Sörensen [1909].

Autocatalysis.

In the hydrolysis of proteins by trypsin, as is well known, a number of amino-acids are set free, and some of these, aspartic and glutamic acids, are fairly strong acids, which will considerably reduce the concentration of hydroxidions in the solution. In fact, as I have recently had occasion to observe, if the initial alkali-content was not very large, say, 2 c.c. of ·880 ammonia to the litre of 10 per cent. caseinogen, the digest becomes actually acid to litmus. This factor must then play an important part in the slowing off of the rate of change in such a case.

The change in hydroxidion concentration during the course of trypsin action has been followed by Brailsford Robertson and Schmidt [1908-9], and found to be capable of expression by a unimolecular formula when the total hydroxidion concentration exceeds 10^{-6}, but by a bimolecular formula when the value is between this and neutrality. Between the two regions there is a short range of transition, where the process follows some formula between the two.

In the case of invertase there is some influence at work which has the effect of accelerating the rate of the reaction as it progresses, since the velocity-constants calculated by the logarithmic formula steadily increase, as shown by Victor Henri [1901]. The explanation seems to lie in some observations by Kullgren [1902], who showed that a similar rise in the velocity-constants took place in the inversion of cane-sugar by water at 100°; in this case the rise was due to the production of an acid as a bye-product, which would increase the rate of hydrolysis. It has not been shown as yet whether in the case of invertase there is any such production of acid, although it is not improbable. Since the action of the enzyme is favoured by small amounts of acid, such a production would explain the increase of "intensity" of the enzyme during the reaction.

According to Hudson [1908] the velocity of reaction of invertase follows the course of a unimolecular reaction. The contrary result of V. Henri is stated to be due to not sufficiently taking account of the multi-rotation of the products of the reaction, especially of glucose, by ensuring that equilibrium was attained when the measurements were made.

Sörensen [1909], however, considers that Hudson attaches too much importance to this factor, and shows that the time-course depends on the concentration of hydrion in the solution. With very low values of this quantity (6·6 to 6·3) [1] there is a considerable increase

[1] These numbers are the exponents of hydrion concentration according to the nomenclature of Sörensen. Their meaning will be clear when it is remembered that distilled water with H · ion concentration of $10^{-7 \cdot 07}$ has the exponent 7·07 and $\frac{m}{1000}$ HCl the exponent 3.

in the value of the velocity constant as the reaction proceeds. With rather higher values (3·68), there is a diminution of the constant, similar to the case of lactase in the experiments of E. F. Armstrong. With a value intermediate between the two (3·92), the velocity constant remains at the same value throughout the reaction. It will be noted that the range of the values of hydrion concentration in these cases lies between that of water and that of 0·001 molar hydrochloric acid.

Phenomena of a similar kind are known in pure chemistry and are called by Ostwald "autocatalysis" [1903, ii., **2**, p. 263]. When an ester is acted on by water the hydrolysis is at first very slow, but as acid is set free the reaction is rapidly accelerated as the acid concentration increases. In an actual experiment it was found that, in the case of a mixture of methyl acetate with water, at 40°, the velocity-constant of hydrolysis steadily increased as the concentration of acetic acid increased, so that at the end of the reaction its value was thirty times as great as at the commencement. This is positive autocatalysis. Other cases are known where the catalyst disappears during the reaction, as in the transformation of oxy-acids into their respective lactones, with disappearance of the hydrion which was acting as catalyst [Henry, 1892]. Such a condition is negative autocatalysis.

Properly speaking, the state of affairs with enzyme is not quite the same as this autocatalysis, where there is production or disappearance of a body which acts of itself as a catalyst. Enzymes produce bodies which are not necessarily themselves catalysts for the reaction, but which act by increasing or decreasing the power of the enzyme itself. The phenomena are sufficiently alike to make it a matter of convenience to use the same name.

Summary.

The various factors affecting the rate of enzyme action dealt with up to the present may now be summed up as follows:—

Causes of retardation:—

1. Reversibility.
2. Combination of enzyme with products.
3. Negative autocatalysis. This, with the previous factor, leads to reversible inactivation of the enzyme.
4. Destruction or similar drastic change in the properties of the enzyme, irreversible inactivation.

Causes of acceleration:—

1. Combination of the whole of the enzyme with the substrate when the latter is in relatively large excess. This leads to

a linear portion of the time-curve, at the beginning of the reaction.

2. Positive autocatalysis.

All of these, with the exception of the first, reversibility, are included in the intensity-factor of Visser. They are of very different relative importance in connection with the various enzymes. The position of equilibrium is only affected by the reversibility-factor; the various components making up the intensity-factor do not cause any change in this equilibrium. When regarded as to their influence on the velocity-constant, as calculated by the logarithmic formula of the simple unimolecular reaction, the retarding causes produce a steady fall in the values, while the accelerating causes produce a steady rise, as in the exceptional case of invertase.

Reactions in Heterogeneous Systems.

In the above treatment of the kinetics of enzyme action, it has been tacitly assumed that the reactions take place in a homogeneous system, *viz.*, in true solution, whereas enzymes, as we know, are colloids, *i.e.*, suspensions of ultra-microscopic solid particles, so that the systems with which we have to do are heterogeneous. It has, in fact, been shown by Dietz [1907, p. 291] that, in the particular case investigated by him, the reaction takes place entirely in the solid enzyme phase. Bourquelot and Bridel [1913, pp. 3-24] have shown that emulsin is able to hydrolyse in 90 per cent. alcohol, in which it is totally insoluble. At the same time, the rate of diffusion of the substrate and products is so great, compared to the rate of the reaction itself, that no appreciable error is introduced by disregarding the diffusion-factor. The reaction, in this respect, is similar to that investigated by Loevenherz [1894], who found that when various esters are hydrolysed by hydrochloric acid in a heterogeneous system of water and benzene, equilibrium of the reacting bodies is very rapidly established between the two phases. The hydrolysis takes place in the phase in which the velocity is the greater, in this case in the aqueous hydrochloric acid, in the case of lipase in the particles of the enzyme. Whether this applies to all enzyme actions cannot be stated with certainty as yet; Arrhenius [1907, p. 142], however, makes the following statement: "The study of the velocities of reactions in heterogeneous systems indicates that they behave very nearly in the same manner as in homogeneous systems. This observation has often been made concerning the velocity of reactions in heterogeneous systems. It depends on the circumstance that by means of the ex-

perimental arrangements the diffusion goes on so rapidly that it does not perturb the chemical processes. If capillary tubes are employed this cannot be said to be the case, and therefore Mett's tubes should not be used for quantitative measurements."[1]

Indirect evidence as to the relatively unimportant part played by diffusion is afforded by the temperature coefficient of enzyme reactions, which is unusually high, as we shall see in the next section. Diffusion, being a physical process, has a low temperature coefficient.

General Equation.

It may serve to bring together the facts of the previous pages if a brief account be given of the general differential equation which Benjamin Moore [1906, p. 83] has put forward for enzyme actions. We will consider that we are dealing with a reaction which is unimolecular in the hydrolytic direction and bimolecular in the reverse direction, such as invertase on cane-sugar or emulsin on salicin. If the reaction proceeded to completion in the former direction and there were no change in the intensity of the enzyme, the velocity of the reaction would be simply

$$\frac{dx}{dt} = K(a - x)$$

where x is the concentration of the products at the time t, K is the velocity-constant, and a is the initial concentration of the substrate, so that $a - x$ is the concentration of the substrate at the time t.

We have seen, however, that the reverse reaction cannot always be neglected, so that we must provide for it in any complete formula. In the case before us it is supposed to be bimolecular, and its velocity will be proportional to the products multiplied together. Since they are present in equal amount, instead of using a separate symbol for each, we may put the square of one, say x^2, and the velocity-constant of this reaction will not usually be the same as the previous one, so that the equation becomes

$$\frac{dx}{dt} = K_1(a - x) - K_2x^2$$

where K_1 is the constant of the hydrolytic process and K_2 that of the reverse process.

Further, some factor must be introduced to allow for the change in intensity, positive or negative, which the enzyme undergoes in proportion to the amount of change that has taken place, *i.e.*, in mathematical language, this factor is some function of the concentration of the substrate, which is $a - x$ for the first member and x for the second. Using

[1] See also p. 121 of the same work.

e as expressing the activity of the enzyme, we may assume as an approximation the factor introduced by Henri, in which the enzyme value is multiplied by a quantity $\frac{x}{a}$, representing the stage at which the reaction has arrived in the one direction and by $\frac{a-x}{a}$ for the reverse reaction. The original $a - x$ must, therefore, be increased or diminished by $e\frac{x}{a}(a - x)$, and similarly for the reverse member. The full equation then becomes

$$\frac{dx}{dt} = K_1\left(1 \pm e\frac{x}{a}\right)\left(a - x\right) - K_2\left(1 \pm e\frac{a - x}{a}\right)x^2.$$

As Moore himself says: "This formula is too complicated for application to experimental results on integration, but it includes all the observed experimental cases, that is, it shows a stage when x is small where the reaction is linear, a stage where the reaction is more rapid than the simple logarithmic law demands, as in Henri's experiments, a stage showing a falling off from the logarithmic values, as in the later stages of Armstrong and of Bayliss, a zero stage at the equilibrium-point, a reversed velocity, which also at the very end tends to become linear".

It would lead us too far to test all the various possibilities of this equation, and the reader must be referred to the work of Moore. Suffice it to say that practically many simplifications can be made, e, for example, can be taken as unity without any perceptible error. In any case it is a matter of some satisfaction that reactions which at first sight are so complex as those in which enzymes play a part, can be expressed in a mathematical formula which is not merely empirical, but in which the several factors have a definite experimental foundation.

We now turn our attention to certain factors influencing the velocity of enzyme action which are capable of definite experimental modification. Such are temperature, initial concentrations of substrate and of enzyme, and addition of various foreign substances such as electrolytes or antiseptics.

Temperature.

As a general rule chemical reactions are increased by rise of temperature in a way that has been formulated by van't Hoff into the well-known rule that for every rise of 10° the rate of a reaction is about doubled or trebled; that is, if a reaction has a rate represented by 2 at 10°, it will become 4 at 20°, 8 at 30° and so on. Put into the form of a curve, this will rise slowly at first and then with increasing steepness until it rapidly becomes nearly vertical.

Enzymes are no exception to this rule, indeed the temperature coefficient for this class of bodies is frequently high. Tammann found for emulsin between 60° and 70° a value of 7·14; I found for trypsin between 20° and 30° a value of 5·3, that is, it took 5·3 times as long to effect the same amount of change at 20° as at 30°.

It is somewhat remarkable that the temperature coefficient of invertase, according to the work of H. Euler and Beth af Ugglas [1910], is actually lower than that of inversion by acids, in fact less than one-half that of the latter. The same statement applies also to the action of lipase and of maltase when compared with the hydrolysis by acid of ethyl butyrate and of maltose respectively.

This effect of heat on the activity of enzymes holds only up to a certain temperature, which varies according to conditions; up to this point raising the temperature increases the rate of change, but a further rise slows the reaction again. This is the phenomenon known as the "*optimum* temperature". Since the property is sometimes regarded as a mysterious one and not shared by inorganic catalysts, it is necessary to examine into its meaning.[1]

Some suggestion as to the explanation is afforded by the experiments of Ernst [1901, pp. 476-77] on the action of Bredig's colloidal platinum on a mixture of oxygen and hydrogen gases. This catalytic reaction shows a temperature *optimum* precisely similar to that of enzymes. The property common to both being the colloidal condition, it is natural to suspect that this, with its sensitiveness to heat, is the cause of the phenomenon in question.

An important series of experiments have been made by Frost Blackman [1905], on the carbon assimilation of the green leaf, which give a complete explanation of the question at issue. It is to be admitted that the chlorophyll function is only in part an enzyme action, but the phenomena are so much alike that there can be no reasonable doubt that what applies to the one applies to the other also. The activity of the process is retarded by the injurious

[1] J. Duclaux [1910, p. 88] even considers it as evidence of "vital action".

effect of temperatures above a certain height, and this by some kind of coagulating action on the colloidal bodies responsible for the reaction. Of what particular nature this destructive action is does not affect the question—the important point being that before complete abolition the process is more or less gradually injured. Here then comes in the importance of the "time-factor," on which Blackman lays much stress, and no doubt correctly. Sachs [1864, p. 116] clearly pointed out that the higher the temperature the more quickly a fatal effect ensued, and that short exposure to a very high temperature may not kill, while a prolonged exposure to a slightly lower temperature was fatal. Now the facts shown in the work on carbon dioxide assimilation referred to above are summarised by Blackman as follows:—

(1) At high temperatures (30° and above for the leaves of cherry-laurel) the initial rate of assimilation cannot be maintained, but falls off regularly.

(2) The higher the temperature the more rapid is the rate of falling off.

(3) The falling off at any given temperature is fastest at first and subsequently becomes less rapid.

This falling off makes it experimentally impossible to determine the highest value at any given temperature, since it is obviously necessary to allow the reaction to continue for a certain time in order to obtain sufficient change to measure it with any accuracy. We can, however, arrive at this indirectly by forming what may be called the van't Hoff curve on the basis of measurements at lower temperatures. Below 25° the rate of assimilation does not fall off with successive estimations, so that by estimations at temperatures differing by 10° we can determine the coefficient for 10°. In the case of the cherry-laurel this is 2·1. The dotted curve in Fig. 5 (from Blackman) gives the calculated initial values for higher temperatures.

Prolonged estimations were then made at higher temperatures, *viz.*, 30·5, 37·5, and 40·5, the rate of falling off in each case being determined. "To plot these on the diagram we regard the base line as having only a time significance, each division representing two hours, and plot out the falling series of readings, obtained at the temperatures mentioned, in curves starting from the initial values indicated on the theoretical van't Hoff curve."

It then becomes at once obvious that the calculated initial value and the observed subsequent values fall into one fairly harmonious curve for each temperature. "We thus attain a graphic demonstration that both methods indicate practically identical initial values, and this" . . . "affords satisfactory evidence that such values actually occur," though

they last too short a time to be measured. At 45° there is a still more rapid fall of assimilation, for which no suitable data are available,

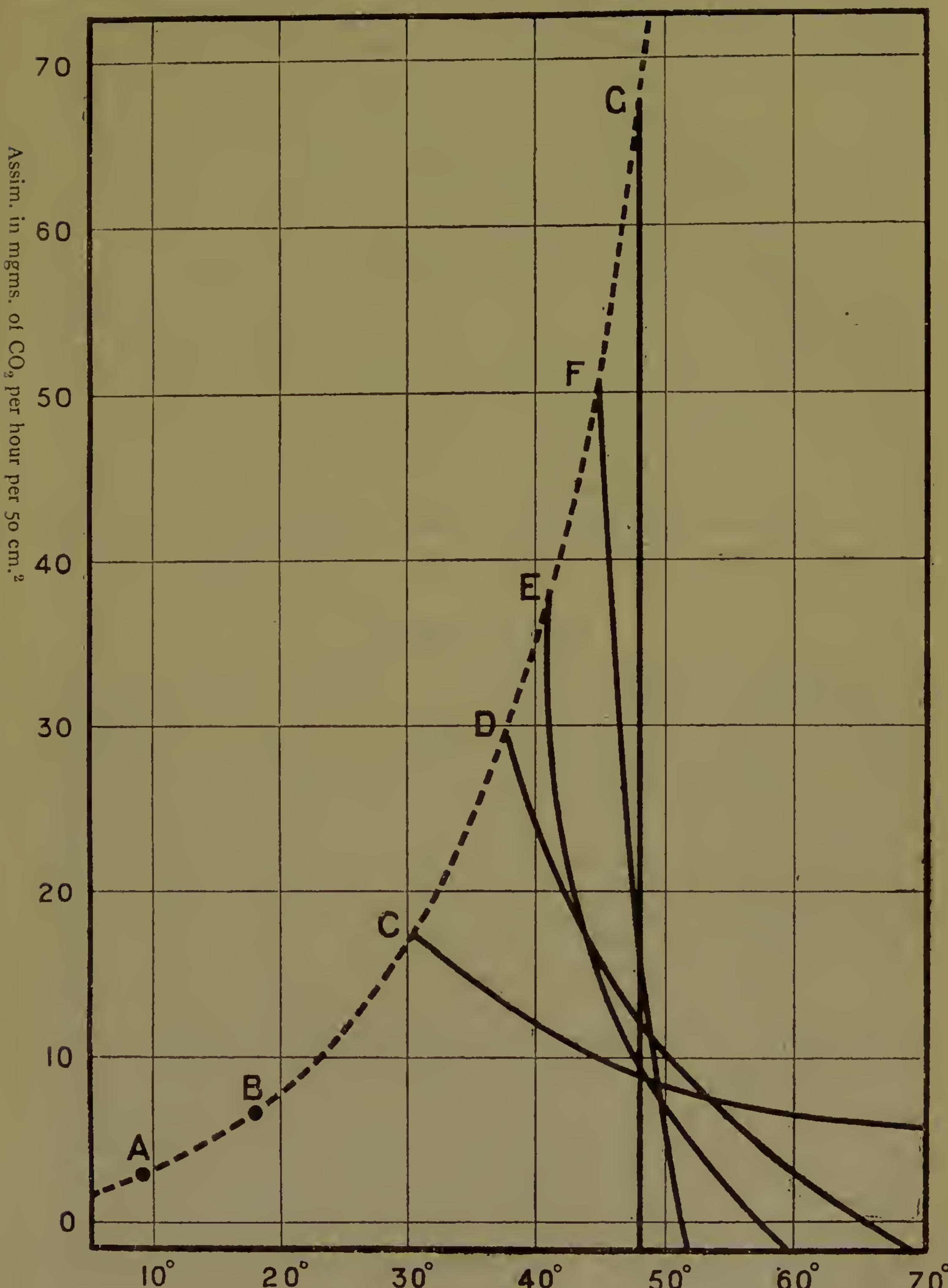


FIG. 5. (From Blackman)

the decline to zero taking place in a very short time. This is the curve starting from F. "Finally, to conclude the series we ought to find a temperature at which the earliest estimation that could be

actually made would give no measurable assimilation. The lowest temperature to give this result might be called the 'extinction temperature,' and here we should hypothecate that, for the first few seconds after attaining it, each chloroplast would give a higher assimilation rate than at any lower temperature, but that the rate would immediately fall, and that so rapidly that it would become *nil* almost at once (say in 100 seconds, for the accepted specific extinction temperature would of course have to be arbitrarily defined in time-units)." This is placed at 48° in the figure, the curve falling vertically from G.

Two things follow from the above results. In the first place the apparent *optimum* temperature will vary considerably according to the time which has elapsed between the beginning of the exposure to a particular temperature and the period during which the estimation is made. Secondly, the so-called *optimum* temperature is merely an expression of the fact that at a certain temperature the increased velocity due to this raised temperature is more than sufficient, for a time only, to counteract the rapid destruction of the enzyme. It has therefore a negligible importance, both theoretically and practically.

Heat Precipitation.

The work of Rettger [1909] on "fibrin-ferment," although it shows that this body is not an enzyme, contains a very suggestive observation bearing on the question before us. The power to excite clotting under certain conditions is usually stated to be abolished by heat. Now Rettger had shown that this happens only when the solution contains protein coagulable by heat, so that, in all probability, the precipitate carries down the "ferment" in a state of adsorption. If the solution thus inactivated by heat be subjected to the action of dilute sodium hydroxide, its activity is regained. The question as to whether enzymes, if obtained free from coagulable impurity, might not be found less sensitive to heat seems worth experimental investigation. I have myself recently tested whether the fact of the greater resistance of invertase to heat in the presence of sugar may not be due to failure of precipitation of the protein impurity in the latter case. The preparation used was a fairly pure one made by Merck, and was not, even in the absence of sugar, coagulated at 65° C., although the preparation used by O'Sullivan and Tompson in their experiments was destroyed at 55° C. in similar conditions. In my experiment, heat coagulation began at about 80° in the absence of sugar, and the solution became opaque at 100°. In the presence of cane-sugar, the solution became merely slightly turbid at 100° and no change was to be detected at 80.°[1]

[1] See also Wohl and Glimm [1910, p. 370].

It does not appear that this possibility applies to all enzymes. Trypsin, for example, loses its activity in alkaline solution without any obvious precipitation. Moreover, if the enzyme in any case is merely carried down with the coagulum, it ought to be possible to regain it by extracting the precipitate, in a similar manner to that of Rettger in the case of "fibrin ferment".[1]

As regards the extinction temperature, it is to be noted that various enzymes differ in their sensitiveness to a raised temperature. It has been already mentioned that it was found by Fraenkel and Hamburg that the "diastase" prepared from malt seemed to consist of two enzymes, as was suggested by Duclaux some years ago. One of these, called by Duclaux [1899, p. 392] amylase, acts upon starch only to such a degree as to convert it into dextrin; the other, dextrinase, is capable of hydrolysing this dextrin to maltose. From the researches of Brown and Heron [1879], and of Kjeldahl [1879], it appears that the dextrinase is more injured by the temperature of 68° than the amylase is. At least this seems to be the probable explanation of the fact that when starch paste is acted on by "diastase" which has been exposed to a temperature of 68° there is less maltose and more dextrin formed than when the enzyme has not been so heated. Reference may also be made to the interesting fact that, in the action of diastase on starch, the reaction ends when the composition of the products is 80·8 per cent. maltose and 19·2 per cent. dextrin. This may be due to a reverse conversion of maltose into dextrin, for, if the mixture be subjected to the action of a mixture of diastase and yeast, although the yeast is only able to ferment the maltose, the dextrin is found to disappear also (A. J. Brown [1904, p. 105]). The reason is that, as the maltose is removed by fermentation, the dextrinase converts a further quantity of dextrin into maltose, which in its turn is attacked by the maltase of the yeast and then fermented.

Maquenne's work [1906], however, suggests that the equilibrium of 80·8 per cent. maltose and 19·2 per cent. dextrin referred to above is due to insufficient activity of the enzyme. If the malt diastase is activated by acid in small amount, the whole of the starch is found to be converted into sugar, so that no dextrin remains. It is probable also that in the fermentation experiment of the previous paragraph the diastase was activated by acid formed by the yeast. In a repetition of the experiment which I made recently, the amount of maltose pro-

[1] E. W. Schmidt [1910] states that trypsin can be boiled, in the presence of peptone or gelatin, without destruction, and Gramenitzki [1910] finds recovery after apparent destruction by heat, but these results have not been confirmed by De Souza [1911] nor by Ohta [1912]. Mellanby and Woolley (communication to Physiological Society), however, find that trypsin in acid solution can be boiled without destruction.

duced in the first stage was greater than that of the equilibrium position of Brown and Heron, perhaps because the action was allowed to proceed for a longer time. The whole was fermented by yeast in the presence of diastase, but the explanation suggested by Maquenne's work was confirmed by the fact that acetic acid was found to have been produced through the agency of the yeast, or other organism associated with it.

Concentration of Substrate.

There is little to be added on this point to what has been already stated. The rate of hydrolysis, by mass-action, should be directly proportional to the concentration, but this actually happens only when the concentration of the enzyme is not too low with respect to that of the substrate. When the latter is in considerable excess, the "combination" of the two bodies produces a state of affairs such that the amount of change is nearly equal with varying concentration of the substrate, as is shown in the table from Frankland Armstrong previously quoted. In some cases, as in that of the action of trypsin on gelatin, the rate of hydrolysis is actually less in the stronger solutions as shown in the following table, which gives in the second column the change of electrical conductivity in twenty-five minutes in solutions of gelatin of the strengths given in the first column :—

Per cent.	Gemmhos.
10	130
8	170
4	240
2	280

This effect is perhaps due to some obscure influence of viscosity. It is not so marked in the case of caseinogen, in which I found the rates of change in the first stages of the reaction to be equal for concentrations of 8 per cent., 5 per cent., and 4 per cent., while that for 10 per cent. was somewhat less than that of 8 per cent.

In the synthesis of glycerol-glucoside by emulsin in my experiments [1913, p. 245], the rate of change was found to be directly proportional to the concentration of the glucose. As regards glycerol, the maximum rate was shown by van't Hoff to be when the ratio of glycerol to water was 4 to 1. In this my results agree with his. I find, however [1913, p. 243], as would be expected from mass-action, that, in equilibrium, more glucoside is present if the glycerol is in higher concentration, although the time taken for the result is longer. This latter fact is, no doubt, due to the physical effect of the viscosity of the glycerol.

Concentration of Enzyme.

This is a question of some importance, since certain results obtained from limited observations have been made the basis of estimations of the relative amounts of enzyme present in solutions.

With regard to the inorganic catalysts, it is usually found that the velocity of the reaction is in direct linear proportion to the amount of the catalyst added. This is not, however, a universal rule.

Various conflicting statements have been made as to the law in the case of enzymes. Some observers have found a direct linear proportion, others that high concentrations are relatively less active than lower. E. Schütz and Borissoff [1885] have gone so far as to formulate a law according to which the action is proportional to the square root of the concentration.

It will be clear, from what has been stated in the previous pages, that these discrepant rules may all be correct, but that they apply to different relative concentrations of enzyme and substrate, or, in other words, to different stages of the reaction, when this begins with relative excess of substrate.

When the enzyme is in considerably smaller concentration than the substrate, the velocity of the reaction is in direct linear proportion to the quantity of enzyme present, owing to the whole of it being able to enter into effective combination with substrate. As the concentration of the substrate diminishes, another law begins to make its appearance, so that the greater quantities of enzyme have relatively less effect. The so-called "law" of Schütz and Borissoff is one particular case of this relationship. What the ratio actually is varies somewhat according to circumstances, but is generally some root less than the square root. The probable meaning of this exponential formula will be discussed in the next chapter. In an actual case in which the question was investigated, *viz.*, in the action of trypsin on caseinogen, the amounts of the trypsin varied in relative amount from 0·5 to 4. The curves of Fig. 1 (p. 18) were drawn from a part of this experiment, and a glance at the slope of the several curves will show that the relative activity of the different concentrations of the enzyme is quite different at the different stages. For instance, between twenty and forty minutes after the commencement of the reaction the slope (= velocity of change) is clearly steeper in curve 2 than in curve 0·5, whereas between 220 and 240 minutes the reverse is the case. It will be seen also that at the same period of 220 to 240 minutes the curves of 4, 2·5, and 2 have the same slope, so that it might appear that the enzyme-concentration was the same in all three.

If we consider the numerical data from which the curves were constructed, the reaction is seen to come practically to a standstill when the electrical conductivity had risen by 2200 gemmhos, so that a change of 800 gemmhos may be taken as representing about one-third of the total. In the first table below, the first column gives the relative trypsin-content, the second the number of minutes from the commencement of the reaction until a change of 800 gemmhos had been attained, while the third gives the calculated times on the assumption of a direct linear relationship:—

Trypsin-content.	Time Observed.	Time Calculated.
4	4·5	5
2·5	7·5	8
2	10	10
1	19	20
0·5	37	40

The substrate here was ammonium caseinogenate in 2·5 per cent. solution. The following is a similar experiment with gelatin in 5 per cent. solution:—

10	2·5	2·67
5	4·8	5·3
2·5	5·9	10·6
1	26·7	26·7
0·5	51	53·4
0 25	90	106·8

The value underlined is obviously an error of experiment. The linear relationship in this early part of the reaction is sufficiently unmistakable.

The next table gives another series of data from the caseinogen experiment, but at a later stage in the reaction, *viz.*, the times taken by the various concentrations of enzyme to effect a change from 1300 to 1800 gemmhos:—

Relative Trypsin-content (c).	Time Taken (t).	Mean Velocity = $\frac{1}{t} \times 1000 = V$.	Specific Activity = $\frac{V}{c}$.	
			Found.	Calculated by Square-root Law.
4	41	24	6	(6)
2·5	48	20·8	8·3	7·6
2	55	18·2	9·1	9·3
1	81	12·4	12·4	12·7
0·5	144	7	14	17·5

A glance at the numbers in the second column shows that the linear relationship no longer holds; in fact, if the mean velocity during the period be calculated, the numbers of the third column, which show that the smaller concentrations are relatively more active than the larger

are obtained. The fact is brought out more definitely if we take the specific activities, *i.e.*, the activity per unit amount of enzyme, as is done in the fourth column. In the last column are given the values given by the Schütz-Borissoff law, which in this stage of the reaction gives fairly good results.

The general conclusion to be drawn from these data is that neither the linear nor the exponential law can be practically applied except when direct experiments with corresponding relative concentrations of enzyme and substrate have shown what law holds. The fact that different observers have formulated different laws is to be explained by their having worked with different relative concentrations of enzyme and substrate or at different stages in the reaction, which both practically come to the same thing.

In the case of trypsin given above, if we had taken as our basis of comparison the times taken to effect a change from the commencement to 1800 gemmhos, it is plain that the law would have been found to be between the linear and the square-root laws. A series of measurements taken with gelatin showed that the ratio was about the 1·5th root, instead of the square root.

Kjeldahl [1879], had already published curves and figures showing this variation of the ratio of enzyme concentration to the activity in different stages of the reaction, *viz.*, first, linear; then exponential, and finally again linear. He also showed that the curve of the velocity of reaction had the same form when the enzyme and substrate were in a certain proportion to one another.

Philoche [1908], in an exhaustive work on amylase and maltase, gives an empirical formula to express the relation of the activity of these enzymes to their concentration as follows:—

$$x = Bc - Ac^2$$

x being the activity, c the concentration of enzyme, B and A constants. This is practically a case of the general exponential law of adsorption which will be discussed in the next chapter.

Hedin [1905], finds that, in the case of trypsin, the effect produced is in direct proportion to the concentration of the enzyme, provided that the substrate is in excess, or, in other words, at the beginning of the reaction. His "time-law," accordingly, states that to obtain the same effect with varying amounts of trypsin, the time of digestion must vary inversely with the amount of trypsin used.

In the case of castor oil lipase, as investigated by Jalander [1911, p. 464], the square-root law is only approximately obeyed.

It is of interest to see whether, in the case of synthetic action, similar relations between enzyme concentration and rate of reaction

obtain, as theoretically, they should do. In my experiments with glycerol and glucose [1913, p. 245] the relative rates with emulsin in the ratios of 1 : 4 : 12 were as 1 : 3, 6 : 6, 3.

For more details with regard to the kinetics of enzyme action, the work of Herzog [1910] can be thoroughly recommended.

The significance of the exponential law will be shown in the next chapter.

Action of Electrolytes.

Enzymes being colloids will naturally be very sensitive to the action of electrolytes. A detailed investigation has been made by Cole [1904, **1** and **2**] on the effect of these agents on the digestion of starch by ptyalin. The action was found to be increased by acids in low concentration and by neutral salts of strong monobasic acids, decreased by larger amounts of acid and by neutral salts of weak acids. On invertase the effects were similar, but not identical. On the whole it appeared that electro-positive ions accelerated, while electro-negative ions retarded. The action was on the enzyme, not on the substrate.

According to Bang [1911], ptyalin is not completely inactive in absence of sodium chloride, although its activity is greatly diminished. The effect of phosphates was also investigated by this observer.

The work of Pavy and Bywaters [1910-11] may be mentioned in this connection.

In some cases enzymes may be said to be inactive in the absence of some particular electrolyte; pepsin is practically so without hydrion,[1] trypsin without hydroxidion. In these cases, therefore, the electrolyte plays the part of a body which we shall learn later to call a co-enzyme.

Michaelis and Davidsohn [1910, **2**] give the optimal $H^{\cdot}$ ion concentration for trypsin as 10^{-8}, that is, the alkalinity of a 0·00000001 molar sodium hydroxide.

Starkenstein [1910, **1** and **2**] has recently shown that the amylase of liver is completely inert without the presence of neutral salts.

Terroine [1910, **1**] finds that the optimal reaction for the lipase of liver is that of 0·007 molar sodium hydroxide.

The action of neutral salts will be again referred to in the next chapter, since it seems to be connected with the facilitation or otherwise of approximation of enzyme and substrate to one another, at least in so far as our present knowledge allows us to form any general view on the question.

[1] J. Schütz [1909], however, states that peptic digestion can proceed without the presence of free acid. What he shows is that a certain amount of digestion can take place when less hydrochloric acid is added than is necessary to make the egg-white used neutral to congo-red. It is not quite correct to state that no hydrion is present.

In certain cases bodies play the part of specific activators, *e.g.*, asparagine on amylase (Effront [1899, p. 143]). A remarkable case of this kind is the relation of papain to prussic acid. Mendel and Blood [1910-11] showed that the proteoclastic action of this enzyme is greatly accelerated thereby. The only substance which has a similar effect is hydrogen sulphide. The result is a puzzling one. It cannot be a case of a specific co-enzyme and the only obvious property shared by these two substances which other weak acids have not, seems to be the possession of reducing properties, which have no apparent connection with the hydrolysis of proteins.

Antiseptics.

A detailed study of this part of our subject does not enter into the scope of the present book, but on account of its practical value in enabling the distinction to be made between the results of the activity of living cells and that of enzymes a few words are required. Emil Fischer has recommended the use of toluene as most appropriate for this purpose [1894, 1895]. It is chemically inert and has scarcely any destructive action on enzymes, while it prevents the growth of protoplasmic structures and therefore excludes phenomena dependent on this. How far portions of cell-protoplasm can be said to be "killed" by it is another question, which is perhaps, at bottom, an idle one, since there are in all probability numerous stages of complexity between an enzyme like invertase or lipase and actual portions of cells, such a stage being zymase. At the same time, so far as a body exerts catalytic actions it is found that it obeys ordinary chemical laws, so that we are justified in treating it as an enzyme.

When the phenomena due to growth make their appearance other laws must be taken into consideration.

Slator [1913] shows that the growth of yeast follows a simple logarithmic law, *viz.*,

$$K = \frac{1}{t} \log_e \frac{N + n}{N}$$

where K is the constant of growth; N the original number of yeast cells, and n the increase in the time, t.

For a detailed investigation of the action of various antiseptics on trypsin the reader is referred to the work of Kaufmann [1903].

It is important to remember that a particular antiseptic may be comparatively harmless to one enzyme and yet very injurious to another. It is impossible in the present state of our knowledge to give any explanation of this fact.

CHAPTER VII.

THE NATURE OF THE COMBINATION BETWEEN ENZYME AND SUBSTRATE.

ABUNDANT evidence has been given to show that, in order that an enzyme may exert its activity, a preliminary combination of some kind between it and the substrate is necessary. Now there are many phenomena, which we have met with in the preceding pages, that assist us in forming a conclusion as to the nature of this combination.

As colloids, enzymes will be particularly prone to form what have been called "adsorption-compounds" (Bayliss [1906, **2**]. Since doubt has recently been expressed by Brailsford Robertson [1908, **1**] as to the actual existence of phenomena of this kind,[1] it is well to repeat what is meant by the use of the name in the present work.

Nature of Adsorption.

The concrete existence of adsorption-compounds can be made visible by an experiment described by myself [1908 and 1911, **2**, p. 83]. It is well known that various colloidal hydroxides, such as those of aluminium, iron, etc., when added to solutions containing colouring matters, will carry these out of solution in the precipitate. Frequent use is made of this fact for the purpose of clarification. If a dialysed solution of the free acid of congo-red, which forms a blue colloidal solution, be treated thus, the substance thrown down will be seen by its colour to contain the blue free acid as such. The undissociated acid is, in fact, blue. The precipitate contains acid and base side by side, as adsorption-compound but uncombined chemically. When it is centrifuged off and suspended in water, no change in its colour will be noticed. But if the suspension be heated to 100° C. combination takes place rapidly, a salt being formed of the characteristic red colour of the congo-red salts. The change also takes place, very slowly, at room temperature. For demonstration purposes, the aluminium hydroxide is the best to use, because the change of colour is more obvious. The hydroxide must not contain free caustic alkali nor free acid. In the former case, the red salt is formed at once, while in the

[1] See also the criticism of Brailsford Robertson's view by van Slyke [1908].

latter case no salt is formed, even on heating. The process is essentially one of mutual precipitation of oppositely charged colloids, since the aluminium hydroxide has an electro-positive charge and the congo-red acid an electro-negative one.

There can be no doubt that there is a condensation of dissolved bodies at the interface between the solvent and the solids suspended in it (Willard Gibbs [1878]). The Gibbs' theorem states that if a body in solution, by concentration on the surface of another phase (*e.g.*, suspended particles of solid or droplets of an immiscible fluid) lowers the surface energy by doing so, the process will tend to take place. This is in accordance with the principle that the energy content of a system always tends to decrease. This principle is known as that of Carnot and Clausius and is involved in the second law of thermodynamics. It is derived from universal experience and never found to be contradicted. Of course, there is nothing inherently impossible in supposing that, in some other part of the universe, beyond our knowledge, free energy might tend to increase. All that we are justified in saying is that it never does so in the universe which we can investigate. Gibbs deduces, from thermodynamical considerations, a formula to express the amount of this surface adsorption, which has been found by Lewis [1910] to give correct values in the case of the condensation of aniline on the surface of mercury, and by Donnan and Barker [1911] for the adsorption of nonylic acid and of saponin at an air-water interface. If, however, the adsorbed body is in colloidal solution, or has an electric charge, other factors have to be taken into consideration. Wo. Ostwald [1909, p. 435, and 195] has in fact generalised the Gibbs' theorem in the following form: Increase of concentration at a surface will always occur when the potential of any form of energy at this surface can be diminished by the process. This includes not only changes of mechanical surface-tension, but also electrical, thermal, and chemical changes. Moreover, the degree of this condensation is frequently in unmistakable relation with the chemical configuration of the bodies concerned. How far it is justifiable in the present state of our knowledge to continue in imagination the process until molecular dimensions are reached is another question.

In certain cases also phenomena due to "solid solution," with slow diffusion in the solid phase, seem to add themselves on and assist in the production of the exponential law of formation of these adsorption-compounds. Such a case is that treated by Travers [1907] *viz.*, the "occlusion" of gases by charcoal.

Davis, also [1907], holds that, in the adsorption of iodine by carbon, there are two factors, a surface *ad*sorption, which takes place

rapidly, and a "solid solution" in, or diffusion into the substance of, the carbon (*ab*sorption). This latter process is slow and arrives at a state of equilibrium only after many days.

It must be remembered that this slow apparent diffusion into the solid may, in both the cases cited, be due to chemical combination, as Freundlich suggests for the case of carbon and iodine [1909, p. 173].

The distinguishing characteristic is just the form of the law which expresses the relation of the composition of the product to the concentration of the bodies forming it; for example, in a concrete case the amount of dye taken up by a stuff is not in direct linear relation to the concentration of the dye, but is relatively greater the lower this concentration is.

In connection with this exponential form of the relation of the amount of adsorption to the concentration of the body adsorbed, it is of interest to recall the fact to which Freundlich [1909, p. 65] directs attention, *viz.*, that the form of the expression connecting the change of surface-tension of a solution with the concentration of the substance dissolved in it has also an exponential form.

Chemical Theories of Adsorption.

It is undoubtedly possible to explain some cases of adsorption by the law of mass-action. But, in order to do this, it is necessary to make additional hypotheses, for which there is no experimental evidence, especially with regard to the number of molecules taking part in the reaction. This number is usually chosen arbitrarily to suit the experimental data and is sometimes fractional. Certain other cases can be explained as being due to partition between the different phases of the heterogeneous system. Here again arbitrary assumptions are made as to the number of molecules which are associated together in the separate phases. Nernst [1911, p. 499] gives an illustration of such a case, and points out the impossibility of any explanation other than adsorption.

The Nature of the Compound between Enzyme and Substrate.

The point of view of the formation of adsorption compounds between enzyme and substrate was put forward by me [1906, **1**, pp. 222-26], and it implies that the changes brought about by the action of enzymes take place essentially at the interface between the phases of a heterogeneous system. We may suppose that, in hydrolysis, for example, the substrate is brought into intimate relation with water on the surface of the particles of the enzyme. This theory is, at the present time, adopted by a large number of workers on the subject. It will suffice to quote the words of Armstrong, Benjamin, and Horton [1913, p. 343]: "The experiments carried out in the course of this series of studies appear to justify the belief that enzymic action takes place entirely at the surfaces of colloid particles suspended in the solution of the hydrolyte and not between substances which are all in true solution". (See also Armstrong, E. F. and H. E. [1913].)

It is to be presumed that all the constituents of the chemical system in equilibrium are condensed on the surface of the enzyme phase, so that the action of the enzyme may be pictured as consisting in the comparatively rapid establishment of equilibrium between them. Of course, the temporary existence of intermediate compounds of a more truly chemical nature between the enzyme, or rather the surface molecules of the particles of the enzyme, and the various constituents of the substrate system is not excluded by the view here advocated, although it must be confessed that we have no evidence as yet as to their existence.

We may take, in illustration, the case of emulsin in alcohol. It was shown by Bourquelot and Bridel [1913, pp. 15 and 54] that emulsin is capable of both hydrolytic and synthetic activities in 90 per cent. alcohol. Now the enzyme is completely insoluble in alcohol of this strength as pointed out by the authors. I have confirmed this fact and shown [1913, p. 251] that 5 grams of emulsin extracted for several days with 75 c.c. of 90 per cent. alcohol gave off no active constituent to the solvent. The filtrate was evaporated in vacuo at 35°, but the residue, dissolved in 2 or 3 c.c. of water, had no action whatever on amygdalin. Prolonged contact with alcohol has no injurious action on emulsin.

Now, when emulsin hydrolyses salicin in 90 per cent. alcohol, it must be able to withdraw from the solvent sufficient water for the purpose. Enzymes are colloids, as we have seen, and in all probability, belong to the emulsoid or lyophile class, which take up water by the process known as imbibition. How far this process is to be

regarded as surface condensation on the colloidal particles or solution in their interior is a matter of dispute. Posnyak [1912] concludes from his experiments on starch that the former hypothesis fits the experimental facts better than the latter, but the relative importance of the two processes probably varies with the concentration of water in the liquid phase. At all events, I found in some experiments with emulsin and with gelatin [1913, p. 252] that both of these take up water from 90 per cent. alcohol, but showed no signs of imbibition; the enzyme particles did not appear to swell, and they were dry and powdery when filtered off from the alcohol, while the gelatin remained brittle; so that, in these cases, the water seems to have been condensed on the surface.

If, then, surface condensation intervenes as a factor in enzyme action, it would be expected that the exponential law of adsorption would be obeyed in the ratio between the activity of an enzyme and its concentration in the solution. It was, indeed, this fact that suggested to me the hypothesis of adsorption. Something of the kind was evidently meant by Victor Henri [1905], although he does not refer to adsorption or surface action. It has also a relationship to Armstrong's view [1891, 1892 and 1895] of the association of enzyme with water and sugar.

That adsorption compounds of enzymes with substrates, distinct from chemical compounds, can be formed is shown by an experiment of my own [1906, **1**, p. 224]. It was shown by W. A. Osborne [1901] that the calcium salt of caseinogen does not pass through a porous clay filter; trypsin, on the other hand, does so. If trypsin is added to a solution of the calcium salt and the solution then filtered through a Berkefeld filter, the filtrate contains no trypsin nor caseinogen. This fact, in itself, merely proves that some kind of compound is formed. If the experiment be repeated with malt amylase in place of trypsin, the filtrate likewise contains no enzyme. It cannot be held that amylase is likely to form a chemical compound with caseinogen. An experiment of Starkenstein, described below, gives similar evidence.

It must be understood that adsorption is merely a preliminary stage, and that, after it has taken place, the proper chemical reaction makes its appearance. The rate at which this reaction proceeds is controlled by mass-action on the part of the adsorbed substances and is, therefore, in proportion to the amount adsorbed. It may be that true chemical compounds with the enzyme are formed temporarily, which, subsequently breaking up again, set free the enzyme for further work.

The mere concentration on the surface of the enzyme in itself will naturally, by mass-action, greatly accelerate a process which is occurring at a slow rate. But it does not seem possible that all the various chemical activities of enzymes can be explained from this point of view alone, although one must be careful in asserting its impossibility.

Further evidence on the question before us will be found to be afforded by consideration of some individual instances. Before passing to these, one or two more general facts may be mentioned.

On p. 26 above, the fact that shaking a solution of rennet renders it inactive was referred to. In further investigations, Schmidt-Nielsen [1910] finds that this is due to adsorption at the interface between air and liquid in the froth produced by shaking. If saponin be added to the solution before shaking, no inactivation takes place. Now it is characteristic of adsorption that a substance which lowers surface energy to a greater extent than another one does will displace this latter from the surface. Saponin has great power of lowering surface energy, possibly by being separated out in a solid form at an interface, so that, if it has already taken possession, rennet cannot be adsorbed.

The Case of Trypsin.

If we refer back to the table on p. 100 giving the times taken by different concentrations of trypsin to effect a change from 1300 to 1800 gemmhos, it will be seen that the lower concentrations are, weight for weight, more active than the higher ones. If we make the reasonable assumption that the rate of change is in proportion to the amount of "compound" of enzyme and substrate in existence at the time, it will be seen that the result is what would be expected if this combination were of the nature of an adsorption-compound, since more trypsin will be in association with the substrate in proportion to the concentration of the enzyme when the latter is lower. The conditions in the first, linear, stage are not inconsistent with this interpretation; in the case of congo-red and paper, when the amount of the dye is very small compared with that of the paper, practically all of the dye is taken up by the paper, since it is necessary to examine the solution in a deep layer in order to detect any colour in it; such an amount would be within the errors of experiment in enzyme work.[1]

The exponential law of activity of enzymes in relation to concentration, of which the square-root law is a particular case, is the mathematical expression of the above facts.

[1] See also the remarks of Denham referred to under the head of "heterogeneous catalysis" below.

That this state of affairs is due to the colloidal nature of enzymes, and therefore an adsorption phenomenon, is indicated by the fact pointed out by Bredig and M. v. Berneck [1899, p. 317] that in the catalysis of hydrogen peroxide by colloidal metals the same kind of law holds, contrary to what obtains in the inversion of cane-sugar by hydrion, where the law of linear proportionality holds.[1]

Further details of this law in relation to adsorption will be found in my paper on adsorption and chemical reaction [1911, **2**, pp. 90-97].

Palladin [1910, p. 364] shows that the relation between the concentration of trypsin and the rate of hydrolysis is only in certain conditions expressed approximately by the square-root law of Schütz and Borissoff; in other conditions, Grützner's law of the two-thirds root is obeyed, that is, the exponent is not one-half but two-thirds. It will be obvious that all these facts are various expressions of adsorption relationships. It cannot be expected that the numerical value of the exponent will remain constant throughout the course of the reaction, since the relative surfaces are continually changing as the colloidal substrate is split up into non-colloidal amino-acids, etc. In fact, in so complex a system, it is not easy to say which component is to be regarded as the adsorbent. Moreover, G. C. Schmidt [1910] has shown that the simple form of the adsorption-equation, given on a previous page, applies only to a limited range of concentration; to include wide differences, a more complex equation is required, in which account is taken of the fact that the surface ultimately becomes saturated, so that further increase of concentration of the solute to be adsorbed does not cause increased adsorption.

An observation by Cesana [1913] shows the importance of surface in enzyme action. Trypsin, exposed to a temperature of 42° for an hour, was found to have an increased activity. Examination under the ultra-microscope showed that the dispersion was greater. The larger surface exposed by this means would account for the rise in the activity.

[1] It has also been shown by Lovatt Evans (*Biochem. Journ.* [1907, **2**, 133-55]) that the square-root law only applies to a limited region in the case of the action of blood catalase on hydrogen peroxide.

The Case of Lipase.

In the experiments of Dietz [1907, p. 314], to which frequent reference has been made, it was found that the velocities of the reactions were proportional to the square roots of the substrate-concentration. A little consideration will show that this is another aspect of the same exponential relationship dealt with above. It was shown also directly that adsorption did actually occur.

Jalander [1911], also, comes to the conclusion that the phenomena observed with castor-oil lipase can only be explained by the formation of adsorption-compounds.

A fact of importance was made out in the experiments of Dietz with lipase, showing that the adsorption-compound of enzyme and substrate is the active system. As the enzyme was in the form of minute particles insoluble in the solution of substrate, the system was heterogeneous, and it was shown in the following way that the reaction took place entirely in the solid phase:—

A mixture of amyl alcohol and butyric acid was acted on by the enzyme preparation. The initial value of the acid was equivalent to 6·50 c.c. of barium hydroxide solution. After two hours forty-six minutes the value was 5·28 c.c. From this mixture 20 c.c. were then removed, filtered, and the clear filtrate, now free from enzyme, put again into the thermostat. After a further twenty-three hours forty-six minutes the original solution had arrived at a value of 1·79 c.c. barium hydroxide, while the filtered solution remained constant at 5·30 c.c.

Berczeller [1911] shows that pancreatic lipase is completely insoluble in fats, fatty acids, and in solutions of fats in ether, so that in this case also the reaction must take place on the surface.

According to Dietz the process probably takes place in the following manner: "The bodies in the solution diffuse into the ferment-phase and are here caused to interact by the agency of the enzyme. The bodies produced in their turn diffuse out into the solution".

But it appears for the various reasons given above (pp. 107 and 108) to be more probable that the action takes place on the surface. Jalander [1911, p. 437] found that the enzyme particles of castor-oil lipase showed no signs of swelling or imbibition in water or dilute acetic acid, nor in pure oleic acid and acetic acid together; but appeared to take up water if neutral fat was present. This author suggests that the fat and water are adsorbed together on the surface of the enzyme and brought into reaction there.

A remarkable fact, which resulted from the experiments of Dietz,

appears to have its explanation in adsorption processes of some kind. It has been already made sufficiently clear that the equilibrium arrived at under the action of lipase is a real one. The same point is reached from both sides and it is independent of the amount of catalyst. It is therefore somewhat unexpected to find that, when acids are used as catalysts, the equilibrium is not the same as that with the enzyme. Under similar conditions, in the former case, *i.e.*, in homogeneous system, the equilibrium is reached with 85·5 per cent. of ester, while in the case of the enzyme the value reached is only 75 per cent. As pointed out by Dietz, this circumstance appears to present an opportunity of evading the second law of thermodynamics, in that heat can be transformed into work at constant temperature by merely changing acid for enzyme and back again in a cycle. But this is impossible unless energy is in some way supplied to the system by the enzyme itself, in which case it would not be in the same state at the end of the reaction as at the beginning. Experiments made for the purpose of detecting changes in the enzyme during the reaction showed that there were none, so that some obscure surface-energy change must in all probability be the cause of the paradoxical behaviour (Dietz [1907, p. 323]).

A similar phenomenon, although in the opposite direction, was observed by Visser in the action of invertase, as referred to in a previous chapter. It is possible that the non-coincidence of the equilibrium-position under enzyme action and under the action of acids is of general occurrence. There are certain facts of importance to be remembered in any attempt to give an explanation of the fact. Although there is this difference in the equilibrium-position in the two cases, it is remarkable that changes in the amount of enzyme do not alter the position in the enzyme system. Another point is that to which Abel has called attention, *viz.*, that, when intermediate compounds are formed, their rates of formation and of decomposition are not necessarily identical, so that, in equilibrium, there may be excess of either one or the other. Again, the fact that enzyme actions take place in a heterogeneous system and the complex phenomena associated with surface development must be remembered. J. J. Thomson [1888] has shown that not only the velocity of a reaction, but also its equilibrium-constant, may be greatly changed in surface films. Ostwald [1908] points out that the equilibrium in hydrolysis of the salt of a fatty acid is altered by the presence in its solution of extensive surfaces. Owing to adsorption of the free acid, the degree of hydrolysis of the salt is increased.

Reference has, in fact, already been made (pp. 7 and 63 above) to the small amount of energy required to change the equilibrium-point in reactions which are practically thermo-neutral, as pointed out by

van't Hoff and by Herzog. It may also be mentioned that A. V. Hill [1911], using a very delicate method, finds that the heat produced in the hydrolysis of starch by amylase is less than 0·5 calorie per gram. When this is compared with the heat of combustion of starch, which is some 5000 calories per gram, we see that it is practically negligible.

Freundlich [1909, p. 522] calls attention to another fact which has to be considered. In the experiments of Dietz, the difference between the position of the equilibrium points under enzyme and under acid was found to be greater, the greater the amount of water present. If the enzyme takes up water by adsorption or imbibition, the water concentration at the place where the equilibrium is actually controlled is probably greater in this case than with acid, where it is uniformly distributed through the system; this excess of concentration of water at the surface of the enzyme would mean a shifting of the equilibrium towards the point of greater hydrolysis, as actually happens.

Falk and Nelson [1912] have shown that amino-acids are also capable of hydrolysing esters, and speak of the action as specific, since some acids hydrolyse esters which are very little attacked by others. The activity is not in immediate relation to their strength as acids, nor does it appear to warrant conclusions as to its having any relation to chemical constitution, since, for example, amino-acetic acid has less action on methyl acetate than phenylalanine has. It is more probably related to some difference in physical properties, such as surface-tension.

The Cases of Maltase and of Amylase.

Mademoiselle Philoche [1908, pp. 355-422] finds that the facts in these instances are most satisfactorily explained by the hypothesis of adsorption between the enzymes and their substrates.

In fact, a definite adsorption-compound between starch and amylase was observed to be formed. A 2 per cent. solution of soluble starch was found to remain unchanged for several days, if left to itself. But, if a small quantity of amylase was added, a precipitate gradually appeared in the course of half an hour or less, according to the amount of enzyme added. This precipitate contained nearly the whole of both the starch and the amylase. The hydrolysis of the starch component took place subsequently.

Van Laer [1911, p. 370] comes to similar conclusions and compares the adsorption of starch and amylase to that of congo-red and filter paper.

Starkenstein [1910, **2**, p. 218] describes an ingenious experiment which shows that adsorption may take place without chemical action. The amylase of the liver is inactive when dialysed free from salts. If such a preparation be shaken with soluble starch at 40°, and afterwards with rice starch in powder, centrifuged and filtered, the filtrate, containing soluble starch and any possible chemical compound of the enzyme with this, if such existed, produces no sugar when sodium chloride is added and the solution warmed. There is, therefore, no enzyme present in any form. The residue, if no sodium chloride be added, also produces no sugar on warming; whereas another portion of the residue, after the addition of sodium chloride, and warming at 40°, is found to contain much sugar. It therefore contained adsorbed enzyme.

Wohl and Glimm [1910, pp. 371-75] show how readily the various phenomena connected with the retarding effect of maltose, etc., on the activity of amylase are explained by the hypothesis of adsorption on the surface of a colloidal catalyst. In certain cases the fact of electrical charges on enzyme and substrate must also be taken into account. ("Electrical Adsorption," see below.)

The fact that sugars have comparatively little effect in lowering surface-tension, and might therefore be supposed to be adsorbed only to a very slight degree, seems to be a difficulty when we have to deal with maltose or cane-sugar as substrates. Michaelis and Rona [1909, p. 492] have shown, however, that the process does actually take place to a not inconsiderable extent, and suggest that changes of compressibility or solubility at the interface may account for the adsorption.

The Temperature Coefficient of Enzyme Action.

The criticism of Brailsford Robertson [1907, **1**, p. 375], that adsorption, as a physical process, has a low temperature coefficient, whereas enzyme action as a whole has the high coefficient of a true chemical reaction, is beside the point, since the chemical changes spoken of above will, of course, have the usual great acceleration by rise of temperature. Caution is, moreover, necessary in making the temperature coefficient a criterion in deciding as to the physical or chemical nature of a reaction. There are cases of chemical reactions which have a small temperature coefficient such as the saponification of ethyl acetate by barium hydroxide at 60°, which has the low value of 1·45 for 10° (Trantz and Volkmann [1908]), while diffusion has a value nearly as high, *viz.*, 1·28. The physical process of imbibition, again, has a high temperature coefficient. Chick and Martin [1910] find that the heat coagulation of proteins has an extraordinarily high temperature coefficient.

A striking instance of the great acceleration produced by rise of temperature is given by Mendel and Blood [1910-11, p. 204]. Papain at 80° digests nearly three times as much excelsin (a plant protein) in fifteen minutes as it does at 40° in seventeen hours; in other words, it is 200 times as active at 80° as at 40°, and has a temperature coefficient for 10° of 3·8.

Heat Protection.

The protection afforded to invertase against the destructive action of heat when cane-sugar is present, as shown by O'Sullivan and Tompson [1890], has been interpreted as evidence of chemical combination between enzyme and substrate. I thought it of interest to test whether mere adsorption is capable of affording any protection of this kind [1911, **2**, p. 96]. The presence of charcoal in a solution of trypsin was, in fact, found to preserve the enzyme to a considerable extent when heated to 60° for ten minutes; one-seventh less was destroyed than in the absence of charcoal, and charcoal is not a very effective adsorbent for trypsin.

Heterogeneous Catalysis.

A few words will at this stage be profitable in explanation of the processes taking place in catalysis in heterogeneous systems. Such reactions, of which those catalysed by enzymes form a part, may be said to take place in three stages. The reacting bodies, *e.g.*, enzyme and substrate, must first of all come into contact, so that their rate of diffusion is the first factor to play a part. The next following process is that of adsorption, which usually takes place with comparatively great rapidity. As we have already seen, the amount taken up in this way is not in direct linear proportion to the concentration in solution. The final process, the chemical change, is conditioned as to its velocity by the amount of the body adsorbed. Possibly in the case of enzyme action, this last chemical process itself happens in two stages, as explained above, an intermediate compound of a chemical nature being the initial stage of the hydrolytic or other change. It is pointed out, however, by Denham [1910], that in only one case of heterogeneous catalysis has a definite chemical compound between catalyst and substrate been shown to exist. In some cases, *e.g.*, colloidal platinum and hydrogen peroxide, there is direct evidence against the formation of an intermediate oxide. This investigator, while regarding chemical combination between enzyme and substrate as possible, considers adsorption to be the chief factor in heterogeneous catalysis, since by

its means the reacting molecules are brought into sufficiently close approximation to enable reaction to take place. He also comes to the conclusion that in enzyme action adsorption precedes chemical change. In all such reactions, the velocity measured is naturally that of the slowest member of the series of stages. It will therefore in different circumstances be controlled by a different member of the series. Since adsorption is rapid, the velocity measured is usually that of either the chemical reaction or the diffusion process. In the case of colloids, the chemical reaction is slower than the diffusion, whereas in metals in mass the diffusion process will be the slower of the two.

Denham also points out how satisfactorily the adsorption theory explains the results of E. F. Armstrong on lactase with varying concentrations of lactose (see pp. 74 and 80). Sugars have a relatively small effect in lowering surface-tension, and will, therefore, in accordance with the Gibbs' theorem, be adsorbed in a rather small degree. The limit of concentration will soon be reached beyond which further increase causes no further quantity to be adsorbed, since the surface-tension would not be lowered to any further degree. Beyond this limit, the amount adsorbed by the enzyme, and, therefore, the velocity of the hydrolysis, will remain constant. In more dilute solutions the adsorbed amount of sugar will be an exponential function of the concentration. The adsorption of caffeine, also, is very like that of sugar, as the concentration is increased a stage is soon reached beyond which a further increase causes no further increase in the amount adsorbed.

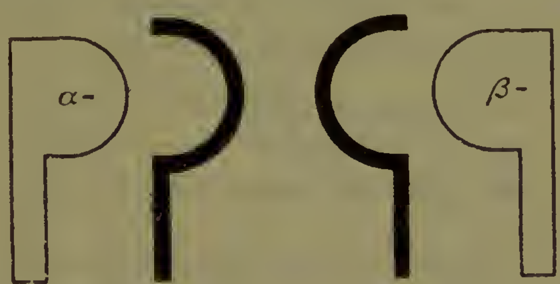
An interesting parallel case to that of enzymes is the disinfecting power of mercuric chloride. Hugo Morawitz [1910], on the basis of the experimental results of Krönig and Paul, shows how the lethal action on bacteria in relation to concentration follows the exponential law of adsorption. The effect of the poison is then proportional to the amount adsorbed. Other cases will be found quoted in my paper [1911, **2**, pp. 84-85].

A somewhat trivial illustration of the phenomena of heterogeneous catalysis may be of service in understanding the process. Take the "reaction" between a strawberry and a number of snails in its neighbourhood. As soon as a snail, in its wanderings, becomes sensible of the presence of the food, it proceeds towards it. This is the first, preliminary, stage of diffusion. The next stage, that of adsorption, may be represented by the attachment of the animal to the strawberry. This takes place rapidly, as soon as proximity is achieved. So long as nothing more happens, no chemical change results. The final stage is the devouring of the fruit and its consequent hydrolysis, etc. This

final stage is obviously dependent, as far as its rate goes, upon the number of snails "adsorbed". It will also be noted that it will not be in linear proportion to the number of snails at work. The more there are, the more they interfere with one another, and, when the fruit is completely covered by them, the advent of more will not further accelerate the disappearance of the food, since the new-comers will not be able to get at it.

Specific Adsorption.

Objection may be taken to the view here put forward, on the ground that no account seems to be taken of the very specific nature of certain enzymes. There is, however, considerable reason for believing that chemical relationship plays an important part in adsorption phenomena. The "lock-and-key" simile of Fischer may be taken to illustrate this fact, so that it may be said that the chemical con-



Maltase. Emulsin.

FIG. 6.

figuration of the surfaces of contact, or the molecular shape of the constituents of the surfaces, are potent factors in determining the possibility of intimate contact between them. Using a somewhat gross illustration, a surface formed of rounded elevations, or projecting spikes, cannot come into close contact with a flat one (see also Starling [1906, p. 40]).

The relation of enzymes to optically isomeric bodies affords support to the view of shape as a factor in enzyme action. This may be seen in Fig. 6, which represents very diagrammatically the relation of the glucosides to maltase and emulsin. Since bodies of three dimensions cannot be readily drawn on a plane surface, the postulate must be made that the figures are unable to move out of the plane of the paper. This being so, it will readily be seen that maltase can enter into intimate contact with the α-glucoside but not with the β-glucoside, whereas emulsin, assumed to be the mirror-image of maltase, can approach closely to the latter, but not to the former.

There is, moreover, actual evidence of a certain degree of specificity in more unquestionable cases of adsorption. It was shown by the pre-

sent author [1906, **1**, p. 213] that gelatin will take up considerably more acid-fuchsin than it will of congo-red, while filter-paper takes up the same amount of both. Gelatin also takes up calcium salts more readily than potassium salts; the same holds for filter-paper, as shown by Schönbein [1861], who found that, when strips of paper were immersed in solutions of various salts, the height to which calcium and barium rose was less than that to which potassium rose, although the height to which the water rose was the same in each.

Zunz [1907] has shown that while some of the proteoses contained in Witte's peptone are precipitated as adsorption-compounds with mastic in the method of Michaelis and Rona [1906, 1907, **1** and **2**], others are not. It can scarcely be held that chemical combination takes place between mastic and these proteins.

Davis [1907] also has shown that, in the simple case of carbon and iodine, specific adsorption makes itself evident. In his experiments it was found that the following relative amounts of iodine were left unadsorbed, under the same conditions:—

By cocoa-nut charcoal	1·236
By bone charcoal	0·522
By sugar charcoal	0·799

This investigator states that his experiments indicate that the surface adsorption is specific, while the "diffusion-factor" is independent of the nature of the surface of the carbon. This fact is significant in connection with what has been said above as to the effect of configuration of surface. The "diffusion-factor" is probably chemical combination with iodine, but the fact would make no difference in the argument.

In the case of the enzymes themselves, a definite case of special adsorption-affinity has been brought forward by Hedin [1907]. Kieselguhr takes up, from a mixture of the two proteolytic enzymes of the spleen, large quantities of the α-protease, leaving the β-protease almost untouched. Charcoal, on the contrary, adsorbs the same proportion of both enzymes. The α-protease, it will be remembered, acts in alkaline solution, the β-protease in acid solution; it is possible that the two bodies have electrical charges of opposite sign and that this circumstance may play a part in the phenomena (Bayliss [1906, **1**, p. 206]). The results of Michaelis [1907] on invertase are also to the point in this connection. This enzyme is adsorbed by certain inert powders, while being left unadsorbed by others.

In the interpretation of apparent cases of "specific" adsorption, care must be exercised against premature unnecessary assumption of

complex chemical factors. The interaction of surface-tension, electric charge, and the various other phenomena at interfaces is capable of an almost endless variety of results. The remarks of Van Bemmelen [1910, pp. 423-30] and of Freundlich [1909, pp. 153-62] should be read in this connection.

Instructive cases of such apparently specific adsorption, but which can scarcely be of a chemical nature, are given in a paper by Wöhler and Plüddemann [1908, p. 664]. Carbon and red oxide of iron adsorb benzoic acid to about an equal degree, but ten times as strongly as they do acetic acid. Chromic oxide adsorbs both acids equally, while platinum black adsorbs acetic acid more than it does benzoic acid, but neither to any considerable extent.

Ultra-microscopic Observations.

Aggazzotti [1907] has recently published some observations on the action of enzymes as seen in the ultra-microscope. These results, according to the author, give support to the theory of combination between enzyme and substrate, although it must be admitted that their interpretation is not easy. It will be sufficient to state here that, at the end of the reaction, certain large granules, few in number, are left unattacked. These bodies which are considerably larger than the particles of the enzyme or substrate, are regarded as consisting of a colloidal-complex of enzyme with certain products of the reaction. The changes which occur on first adding the enzyme to the substrate are apparently too complex to enable any conclusions to be drawn whether union of these two bodies took place.

Jerome Alexander [1910] has made the following observation upon the action of diastase on potato starch grains under the ultra-microscope. "Actively moving ultra-microns in the diastase solution gradually accumulated about the starch grains, which after a time showed a ragged and gnawed margin." Until we know more as to the meaning of the phenomena seen by the method, considerable caution must be exercised with regard to their interpretation.

The observation of Cesana [1913] on the change in the dispersion of enzymes, referred to above under the head of "Trypsin," is an illustration of the use to which the ultra-microscope may profitably be put.

Solubilities of Enzymes.

The solubilities of some enzymes indicate a relationship to their respective substrates. As shown by Dietz [1907, p. 286], pancreatic tissue can be washed free from trypsin by water, which leaves the lipase behind. Lipase is, however, readily soluble in glycerol, and has been stated to be soluble in ether, or rather in an ethereal extract of liver, probably containing lecithin (Ramond); the apparent solubility was, no doubt, due to adsorption by lecithin. (See the work of Loewe [1912]).

Action of Electrolytes.

Reference has been made in a previous chapter to the effect of electrolytes on adsorption. If the enzyme and substrate are colloids and both with an electric charge of the same sign, it is plain that a certain mutual repulsion will tend to obstruct the formation of a compound, just as in my experiments negative paper takes up very little negative congo-red, but when given an opposite charge by a kation, a large amount of the dye is adsorbed. Similar phenomena are to be seen when the electro-negative colloid, arsenious sulphide, is taken in place of congo-red. It seems then very probable that the action of electrolytes on enzymes may in some cases be referable to this circumstance. As yet the question awaits investigation. Trypsin is stated by Victor Henri to be electro-negative [1]; in agreement with this I have found that the amount of it which is taken up by paper is increased by the presence of calcium sulphate [1906, 1, p. 226].

Further experiments were made in 1911 (p. 88) to compare the adsorption of this enzyme by different electro-negative surfaces, in presence and absence of an electrolyte. Adsorption by caseinogen, starch, and filter-paper was increased in all cases by the presence of calcium sulphate. It is interesting to note that filter-paper adsorbed more trypsin than its appropriate substrate, caseinogen, did. Michaelis and Ehrenreich [1908] find that malt diastase in alkaline or neutral solution is adsorbed only by electro-positive aluminium hydroxide, not by electro-negative kaolin. Its charge can, however, be reversed by acid, in which case its behaviour with respect to the above adsorbents is also reversed. It is interesting to note that calcium ions have been shown by Pottevin [1906] and by Kanitz [1905] to increase the activity of both trypsin and lipase; their action is therefore not specific, but is probably due to their effect on adsorption of substrate by enzyme. This effect of kations on trypsin is not obtained except in very low concentrations of the electrolytes; in higher concentrations they are

[1] See Iscovesco [1906].

injurious. This fact is again in complete agreement with the facts of adsorption, as seen in the case of congo-red and paper. Here also if the concentration of the calcium is greater than about 0·005 molar, the dye is precipitated in such a way that the large particles are not taken up by the paper at all; the colloid must not be caused to agglomerate or the adsorption will not take place.

The importance of electrolytes in facilitating the necessary adsorption of enzyme and substrate is shown by the following observation of Philoche [1908, p. 393]. Dialysed pancreatic juice has no action on starch, as shown by Bierry, Giaja, and Henri [1906]. No precipitate is formed in this case, but, if sodium chloride be added, the adsorption compound of enzyme and starch is thrown down and hydrolysis commences.

It is stated by Iscovesco [1910] that pepsin is electro-positive, even when dialysed. The charge disappears, on the contrary, when its solutions are boiled. The measurement of the isoelectric-constant by Michaelis and Davidsohn has been referred to above (p. 27). Pekelharing and Ringer [1911] give reasons for doubting the value of this constant as applying to all preparations of pepsin.

On pancreatic lipase the action of electrolytes is of a double nature, similar to that described by Cole in the case of invertase. A detailed investigation has been made by Terroine [1910, 5], but no complete explanation was found.

Experiments on the effect of electrolytes on the value of the exponent of the adsorption equation were made by myself in 1910. In the case of congo-red and filter-paper, I found that the value of n is increased by the presence of electrolytes, so that its value becomes nearer to what it would be in a chemical reaction with precipitation, *i.e.*, infinity. Corresponding experiments with trypsin [1911, **2**, p. 94] gave a similar increase in n. Put in other words, there is less difference between the activity of different concentrations of enzyme in the presence of calcium sulphate than in its absence. At the end of the reaction, an opposite result was obtained, so that the total effect of electrolytes must be regarded as a complex one.

On the whole it seems evident that the action of electrolytes may be due to very various causes in different cases, so that it is impossible to formulate statements of general application. In the next chapter it will be seen that some enzymes are quite inactive without the presence of electrolytes.

"Electrical Adsorption."

A few more details seem desirable here in regard to the process sometimes known by this name. The investigations by myself referred to above [p. 30] were the first systematic experiments on the subject. There are two ways in which electrical charges may play a part in adsorption. In the first place, if the sign of the charge on a surface is of opposite sign to that of ions or colloidal particles in the liquid phase, it is plain that the deposition of these latter on the surface will be facilitated. The contrary will be the case when the charges are of the same sign. Influences which diminish, or change the sign of, a charge will therefore have considerable effect on the degree of adsorption.

In the second place, processes which diminish the potential difference between the phases will, by an extension of Gibbs' theorem, tend to take place, since by so doing, the total energy of the system will be lessened. One may therefore state it as a general rule for the action of electrolytes, that those ions which raise the potential difference between adsorbent and body adsorbed will act favourably on adsorption; whilst, on the contrary, those ions which cause a diminution of potential difference will hinder adsorption. For further explanation the reader is referred to Wo. Ostwald [1909, pp. 422, 433].

Various facts relating to the importance of electrical adsorption in enzyme action have been given in the preceding section.

Zymoids.

There are some facts which give support to Ehrlich's view that the combining power and fermentative activity are functions of distinct "side-chains". It was found by Korschun [1902], when investigating the relations between rennet and its anti-body, that by filtration through porous clay a solution of rennet could be separated into several fractions which, by appropriate dilution of the stronger fractions, could be brought to the same strength as regards combination with the anti-body, but which differed considerably in their power of clotting milk. In other words, the original solution appeared to contain a modified form of the enzyme analogous to Ehrlich's "toxoids"; that is, a part of the enzyme had lost its characteristic action while retaining its power of combining with the anti-body. I have myself [1904, p. 271] met with some facts which point to the production of a similar modification of trypsin by warming to 25° for a day or so. I suggested calling these modified enzymes "zymoids". The experiments of Bearn and Cramer [1907] are also of interest in this connection.

It seems probable, from the various facts to be mentioned below under "Anti-enzymes," that the phenomena connected with "zymoids" are cases of adsorption by enzymes which have lost their activity.

CHAPTER VIII.

CO-ENZYMES AND ANTI-ENZYMES.

Lipase.

It was noticed by Magnus [1904] that, when an extract of liver was subjected to dialysis, the lipolytic power which it originally possessed was gradually lost, but was regained when the dialysate was added. This experiment shows that what may be called the lipoclastic system of the liver consists of more than one component, each of which is separately inactive. The inactive dialysed extract prepared by Magnus was also restored to activity by the addition of boiled liver-extract, or by a similar extract from which proteins had been precipitated by uranyl acetate. The activating body was soluble in alcohol, but not in ether, and was not present in the ash of liver. The component which did not dialyse was destroyed by boiling and may therefore be regarded in a sense as the enzyme proper, while the dialysable, thermostable body, or bodies, may be called the "co-enzyme".

Laccase.

This name "co-enzyme" or "co-ferment" was introduced by Bertrand [1897] to express the great increase in the oxidising power of laccase brought about by the addition of manganese salts in minute quantity. It has not, however, been shown that laccase is actually inactive apart from manganese, so that the original use of the name "co-enzyme" was rather in the sense of what we now sometimes call an "accelerator," similar to asparagine in connection with amylase, as already mentioned. Bertrand also applied the name to calcium salts, in the case in which they are apparently necessary for the action of pectase on pectin.

Zymase.

Harden and Young [1906, 1 and 2, 1908, 1909, and 1910] have described a case of well-marked co-enzyme relationship in the alcoholic enzyme of yeast-juice. When yeast-juice, prepared by Buchner's method, is filtered under pressure through a Martin's gelatin filter, the colloids are left behind on the gelatin. The substance so obtained, which would be expected to contain the enzyme, showed itself to be inactive, but when mixed with a portion of the filtrate, which, alone, is equally inactive, it became capable of exciting vigorous fermentation. The co-enzyme found in the filtrate is dialysable and not destroyed by boiling. It disappears from yeast-juice during fermentation and when the juice is allowed to undergo autolysis. The evolution of carbon dioxide from a mixture to which a small amount of co-enzyme has been added soon ceases, but can be renewed by the addition of more co-enzyme. As to the nature of this body our knowledge is as yet incomplete. In view of the fact that soluble inorganic phosphates are able to greatly increase the activity of an ordinary yeast-juice, it was thought that these substances might be the co-enzyme. Experiments showed, however, that the inactive residue could not be brought back to activity by this means, although subsequent addition of boiled juice was able to do so. Moreover, the boiled autolysed juice does not set up fermentation in a mixture of the inactive residue with glucose, although it contains a large amount of soluble phosphate. Subsequent work (Part III.) showed that both the bodies mentioned are necessary for the activity of zymase. The system is, therefore, a very complex one, two co-enzymes, in fact, being required. Phosphates are necessary, but ineffective apart from the presence, in addition, of another co-enzyme whose nature is, as yet unknown.[1] "The cycle of changes which the phosphate undergoes appears to be the following:—

(1) $2C_6H_{12}O_6 + 2Na_2HPO_4 = 2CO_2 + 2C_2H_6O + C_6H_{10}O_4(PO_4Na_2)_2 + 2H_2O$

(2) $C_6H_{10}O_4(PO_4Na_2)_2 + 2H_2O = C_6H_{12}O_6 + 2Na_2HPO_4$."

These changes, of course, take place under the influence of the enzyme-system.[2]

[1] According to Buchner [1909] the co-enzyme is probably an ester of phosphoric acid.

[2] See Harden's *Alcoholic Fermentation* in this series.

Bile-salts, the Co-enzyme of Lipase.

The chemical nature of the lipase co-enzyme is better known than that of zymase. Loevenhart [1905] showed that bile-salts possessed all the properties of the co-enzyme, while v. Fürth and Schütz [1907] have shown that sodium cholate is as active as sodium glycocholate.

Magnus [1906] also showed that synthetic bile-salts have the same action as the natural bodies. The importance of this fact is that it shows that, unlike the action of phosphate on yeast-juice, no additional co-enzyme is required, such as might possibly be contained in preparations of the natural bile-salts.

It appears that bile-salts do not act entirely by facilitating emulsion of insoluble fats, since the hydrolysis of esters which are soluble in water is also accelerated by them. (See Terroine [1910].) This same investigator, at a later date [1910, 4] came to the conclusion that the action is exerted on the enzyme itself. He found that bile-salts, acting on pancreatic juice alone, activated it very rapidly, but if the action is more prolonged, the lipase is destroyed. The rate of destruction is proportional to the concentration of bile-salts, so that it does not appear that spontaneous inactivation is concerned.

It is possible that the effect on the enzyme is somewhat of the nature of a prevention of colloidal aggregation of it, or production of disaggregation, owing to the great lowering of surface-tension produced by bile-salts. In this way a larger active surface is ensured.

Rosenheim and Shaw-Mackenzie [1910] find that serum has also an accelerating effect on lipase. The same fact was noticed by Beitzke and Neuberg [1906].

An important point as regards the theory of synthesis by enzymes was discovered by Hamsik [1910]. If bile-salts accelerate hydrolysis, it would be expected that the synthetic process would also be accelerated. This was, in fact, found to be the case with the synthesis of triolein by the lipase of the pancreas and of the intestine. According to Terroine [1910, 2], however, the two processes are not equally accelerated, since the equilibrium-position is shifted towards the side of greater hydrolysis in the presence of bile-salts. After 926 hours, there was found to be nearly twice as much hydrolysis in the digest containing bile-salts than in that without this addition. It is not quite clear, however, whether in the latter case, the enzyme was still active, or had disappeared or become inactive, before its work was done.

Dialysis of Pancreatic Juice.

One more instance may be referred to. It has been shown by Bierry, Giaja, and V. Henri [1906] that if pancreatic juice be dialysed it loses its power of acting upon starch or maltose. The addition of certain electrolytes restores this activity. By testing various salts it was shown that the electro-negative ion is the only potent one, and among these ions the chlorine or bromine ion is the essential one. Thus, sodium and potassium chlorides are active, while the sulphates are inactive.

Starkenstein [1910, 1 and 2] shows that amylase from the liver becomes inactive when dialysed and that its activity is restored by sodium chloride. He notes that neutral salts appear to facilitate the effect of hydrolysing enzymes, but not that of oxidising ones.

It was thought at one time that malt amylase could not be made inactive by removal of electrolytes, but Vulquin and Lisbonne [1912] have shown that mere dialysis is incapable of doing this effectively. In combination with electrolysis, which carries the ions through the membrane, the enzyme can be deprived of activity by dialysis. The activity is restored by the addition of chlorides of potassium, sodium, or calcium and by potassium nitrate.

"Anti-enzymes."

The question as to the existence of anti-enzyme in the true sense, as used in the theory of immunity, is a somewhat difficult one. But, since completely unwarranted assumptions as to their production are repeatedly being brought forward in explanation of the most various experimental facts, a few words are necessary.

An anti-body is a substance of a complex nature produced by the injection into an organism of some other substance (its antigen) and acting specifically by preventing the action of its own antigen and not that of any other. It is destroyed by heat. As to the nature of the substances capable of acting as antigens, there is no doubt as to the fact that any foreign protein is such. Statements have been made that certain complex lipoids and glucosides can act as antigens, but the evidence that they were free from protein is not very satisfactory. Antigens, in fact, belong to the class of substances of high molecular weight regarded by Ehrlich [1902] as analogous to food-stuffs and taken up in some intricate manner by the protoplasmic molecules. The other class of substances act by the chemical or physical character of their molecules and do not give rise to anti-bodies when injected. Such substances are of comparatively small molecular weight; most drugs and the chemical messengers or "hormones" belong to this class.

The facts that enzymes are not proteins and that evidence is accumulating to show that their chemical constitution is of a simpler nature than was supposed at one time, are, *prima facie*, grounds for doubting their capacity of acting as antigens. Moreover, my negative experience with emulsin as briefly referred to above (pp. 61 and 62) caused me to examine the evidence brought forward. It was in this actual case of emulsin that the first "anti-enzyme" was described by Hildebrandt [1893]. The serum from animals "immunised" to emulsin inhibited the action of emulsin on glucosides. I have already shown [1912, p. 460] that this effect is merely due to diminution of the hydrogen-ion concentration of the reacting system. This fact, in itself, has not been sufficiently taken into account. Enzymes are extremely sensitive to hydrogen-ion concentration, and many "anti" effects of serum are due to changes in this factor alone. In recent experiments by Ohta [1913], the serum from rabbits immunised by emulsin was found, in some instances, to have a greater inhibiting effect on emulsin than normal serum has. But, as no account was taken of possible differences of $H^{\cdot}$ concentration, the results are not convincing. Further evidence given by Hildebrandt is that in normal rabbits emulsin produces inflammation at the seat of injection, whereas it does not do so in rabbits previously immunised. In my experiments, no irritative effect was ever produced, so that I must conclude that the effect in Hildebrandt's experiments was due to something other than emulsin, probably a foreign protein, to which an anti-body would be formed. The only other evidence brought forward is derived from the results of injection of emulsin on the action of certain chemical substances. The conditions in such experiments are very complex, and the results can more easily be explained as due to synthetic action on the part of the enzyme itself, rather than as due to synthetic action of anti-emulsin, as stated by the author [1906]. Similar considerations apply to the results of Hämäläinen and Sjöström [1910]. It seems more probable, however, that they had no connection with the injections of emulsin.

I have already pointed out that many of the "anti" effects shown by serum can be accounted for by change of reaction, but this fact does not seem capable of explaining the increase of such effects stated to be produced by injection of enzymes. It is to be remembered, however, that when the normal blood already shows such properties, it is practically impossible to be certain that an increase following an injection is not due to a spontaneous change. Natural variations are, in fact, very considerable.

A further fact to be kept in mind is that substances capable of

taking up enzymes by adsorption produce a diminution of their action merely by reducing their concentration. A very marked effect of this kind is that of charcoal on trypsin, as investigated by Hedin [1906]. It is found to obey all the characteristic laws of adsorption ; a specially interesting fact being that saponin, as shown by Jahnson-Blohm [1912] prevents the adsorption and anti-action in a similar way to that in which it prevents the inactivation of rennet by shaking. The "specificity" of anti-enzymes does not appear to be very marked, and we have already seen that there are a great variety of possibilities in adsorption which may simulate specific action. This effect of adsorption in removing enzyme from the sphere of action is sometimes, unnecessarily, called "deviation," as by W. W. Hamburger [1911].

An anti-tryptic action of serum has been shown by Cathcart [1904] to be associated with the albumin fraction of the proteins, not with the globulin fraction, as is usual with true anti-bodies. It seems probable that the effect is due to adsorption of the enzyme, thus diminishing the effective concentration. Hedin showed [1906] that the whole of the trypsin cannot be rendered inactive by it and that small amounts are relatively more active than larger ones, thus it behaves quite in the same manner as charcoal. The effect of heat in abolishing its activity might, at first sight, be regarded as an indication of its nature as being that of a true anti-body, but the result might well be accounted for by the changes in physical state, diminution of surface, etc., involved in coagulation.

According to Rosenthal [1910] the anti-tryptic action of serum is really due to products of proteoclastic action, but the experiments are not very convincing.

When raw serum or egg-albumin is acted on by trypsin, it is found that no effect appears to be produced for some hours ; gradually the enzyme begins to act and regains its power in such a way that the curve of the rate of action is convex to the axis of abscissæ. Fig. 7 shows this fact in the lower curve (B). The egg-white was diluted with nine times its volume of water. The upper curve (A) was given by a similar experiment in which the egg-white had been previously heated to 100°. It will be noticed that the two curves finally arrive at the same point. It seems evident that the explanation lies somewhat as follows. The raw protein, for some reason not as yet clear, is difficult of attack, but adsorbs the enzyme. As it is slowly attacked and converted into products which have no adsorbent properties, more and more of the enzyme is set free to act.

Coagulated albumin is known to be more readily attacked than in its native condition.

There is no doubt of the existence of substances which have a markedly inhibiting action on certain enzymes, although it leads to confusion if they are called "anti-enzymes," since there is no evidence that they can be produced in response to the injection of these enzymes into organisms.

Such a substance is that found by Weinland [1903, 1] in intestinal worms; these organisms are protected from the action of the pancreatic juice of their hosts by this means. The properties of this substance were investigated by J. M. Hamill [1905-6]. It is not destroyed by boiling in neutral or acid solution; but, if even slightly alkaline, it is immediately destroyed. It is soluble in alcohol of any

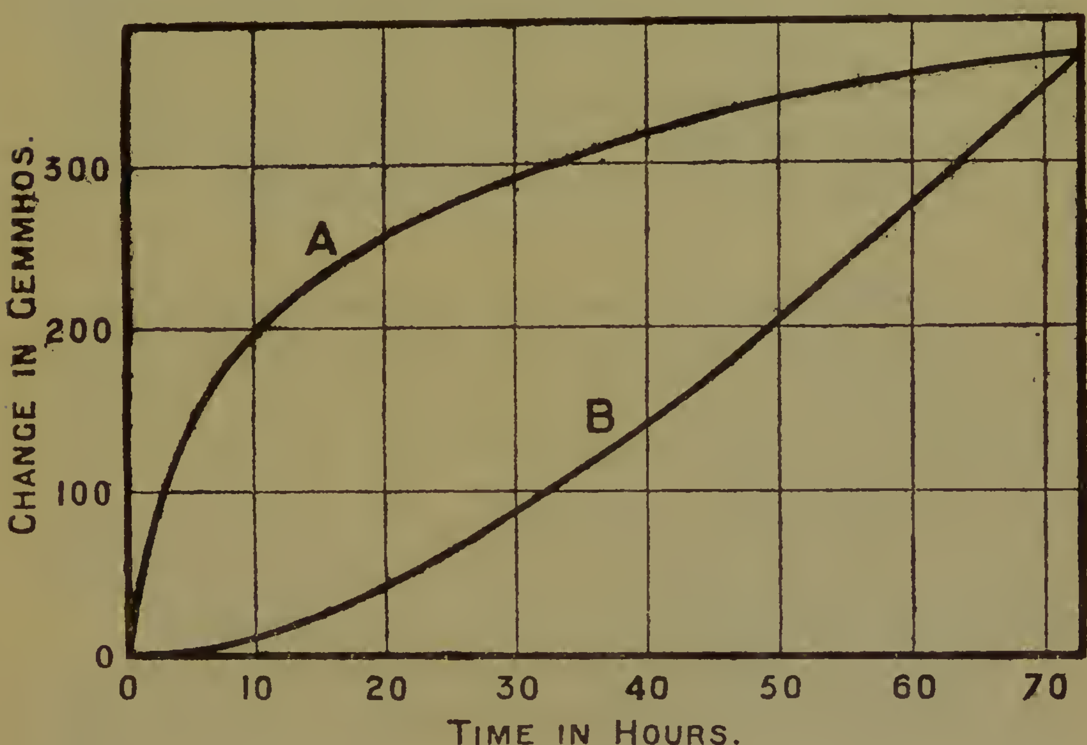


Fig. 7.

strength below 85 per cent. and it dialyses through parchment paper. It is obviously a very different substance from an Ehrlich anti-body. It is slowly destroyed during the course of a tryptic reaction to which it has been added, as Dastre showed [1903], a fact which I have also been able to confirm. If a mixture of trypsin and caseinogen, to which sufficient worm-extract has been added to prevent action, be allowed to remain at a warm temperature for a few days, activity is regained. Since the inhibitory substance is destroyed in alkaline solution by boiling, it is most likely to be slowly acted on at the temperature of the digestion.

A somewhat similar substance is described by Buchner and Haehn [1910] as being present in yeast. This is believed to act by combination with the substrate, rather than by action on the enzyme. It protects zymase and also caseinogen and gelatin from the action of the endo-tryptase of the yeast. Gelatin is also protected from the action of pepsin and trypsin. This inhibitory substance is not destroyed by boiling and gives no protein reactions, but is destroyed by the action of lipase. The authors regard it as an ester of some organic acid.

Hedin [1912] describes the production of an anti-rennet by the injection of either rennet or its zymogen. He points out that it is not an anti-body in Ehrlich's sense, since the injection of zymogen already containing the anti-body causes the production of a larger amount of the substance than the rennet itself does. The anti-body of the zymogen apparently itself affords material for the formation of the anti-body in the blood. The conditions are very complex and the original paper must be consulted by those interested.

Protection of the Intestinal Mucous Membrane.

Weinland [1903,] has stated that anti-pepsin exists in the gastric mucous membrane and anti-trypsin in that of the intestine; these bodies are supposed to confer upon these tissues their immunity from attack by the digestive juices. According to some unpublished work of Hamill as well as of myself, the existence of anti-trypsin in the intestinal mucous membrane is very doubtful, although Hamill confirms the presence of anti-pepsin in the gastric mucous membrane.

According to Klug [1907], the actual body which has the power of protecting the cells of the mucous membrane is the mucin which is always present in considerable quantity. This it does by forming adsorption-compounds with the enzymes.

If this be so, it may reasonably be extended to the other colloids present in the foods taken into the alimentary canal. It is to be presumed that, by mass-action, the greater part of the enzymes is in combination with these substances. It is certain that, if a copious secretion of pancreatic juice is poured into the empty intestine in consequence of the injection of secretin, great damage is done to the mucous membrane, resulting in desquamation of the cells and hæmorrhage. No doubt, under normal conditions, the acid gastric contents, which serve to excite the secretion of the pancreatic juice, when they arrive in the duodenum, also effect a partial neutralisation of its

strong alkalinity, a factor which is absent when the pancreatic secretion is excited by the injection of ready-made secretin. At the same time, it is obvious that the reaction must remain sufficiently alkaline in order that the trypsin may exert its action. It seems probable, then, that the injurious action of pancreatic juice on the empty intestine may be, in part, due to the absence of food-stuffs, which would take up the enzyme.

CHAPTER IX.

ZYMOGENS.

The relation between enzyme and co-enzyme is a reversible one. This can be seen by considering the case of the liver lipase. The extracts were at first active, became inactive on dialysis, but regained activity on the addition of bile-salts.

The relation of inactive zymogen to active enzyme is an irreversible one. Since all enzymes are produced by the agency of living protoplasm, it is evident that at some stages in their formation they must be devoid of the catalytic properties of the fully formed enzyme. This stage is called a "zymogen" when it can be obtained free from the cells in which it was formed and can be converted by purely chemical means into the active enzyme. When this change has taken place the new body cannot, as far as we know, be reconverted into the zymogen.

Trypsinogen.

The trypsin of the pancreatic juice is actually secreted in the form of a zymogen and is poured into the duodenum in this state, as has been shown by the present author in conjunction with Starling [1902, p. 347]. In the duodenum it meets with the enzyme, enterokinase, and is converted by this into active trypsin. Except for the fact of its being devoid of proteoclastic power, trypsinogen, as we may call the zymogen of trypsin, has properties very like those of the enzyme.

Recent experiments of Delezenne [1905] show that inactive pancreatic juice can be activated by calcium salts as well as by enterokinase. The amount of calcium present in the juice as secreted is sufficient to bring about very slow activation, but the process can be considerably accelerated by adding more calcium. These results have been confirmed by Miss B. Ayrton [1909], who states that spontaneous activation of pancreatic juice occurs, apart from the addition of enterokinase, or other external agent. This is dependent upon the presence of calcium salts together with some other factor, which is destroyed when the calcium salts are removed by precipitation. It is

not possible, in fact, by the addition of calcium salts, to activate juice which has been decalcified.

According to the detailed researches of Mellanby and Woolley [1912 and 1913], activation is in all cases produced by enterokinase. They hold that enterokinase is a proteoclastic enzyme, acting best in neutral solution and easily destroyed by alkali. Trypsinogen is a compound of a protein with trypsin, and, when acted upon by enterokinase, the protein is digested and the trypsin liberated. It is not quite clear, however, why the protein moiety of the compound is not digested by the trypsin moiety, if it is attacked by another enzyme. The authors believe that the activation on addition of lime salts is really due to neutralisation of the juice by precipitation of calcium carbonate and formation of sodium chloride. In consequence of this, the traces of enterokinase, which are derived from the pancreas itself and are contained in most tissues, enter into activity. As enterokinase is extremely sensitive to alkali, the juice as secreted causes the rapid destruction of the enterokinase contained in it and, accordingly, does not become active spontaneously. There appears to be some substance present in serum which has an antikinase action, but, since the test used in the experiments of these authors was the clotting of milk, a colloidal phenomenon, no conclusion can be drawn as to whether this was a true anti-body or whether the result was due to adsorption of enterokinase by some other colloid.

Pepsinogen.

The first preparations containing a zymogen in solution were those of Langley and Edkins [1886]. These solutions contained a subtance, pepsinogen, which, on treatment with hydrochloric acid, was converted into active pepsin. The proof of the existence of a zymogen in extracts containing pepsin itself was rendered possible by the discovery of Langley [1880-82, p. 253] that pepsin is much more rapidly destroyed by alkali than is pepsinogen. The latter is very rapidly converted into pepsin by acids; at 20° all or nearly all the pepsinogen present in an aqueous extract of a cat's gastric mucous membrane may be converted into pepsin in sixty seconds by 0·1 per cent. hydrochloric acid.

Pepsinogen has been prepared by Glaessner [1902] in what appears to have been a nearly pure solution by a method of which the following is an outline. The mucous membrane of pigs' stomachs was allowed to autolyse in alkaline solution at 40° for some weeks. Mucin was removed by precipitation with acetic acid and proteins

with uranyl acetate; this last precipitate carried down with it, in a state of adsorption, the pepsinogen. By extracting the mass with a large amount of dilute sodium carbonate the greatar part of the zymogen passed into solution again. The process of uranyl acetate precipitation and extraction of precipitate with dilute sodium carbonate was repeated and the final extracts concentrated at 40°.

The body so obtained gave only two of the protein reactions, and these very faintly, namely, a fine precipitate with mercuric salts and with phosphotungstic acid. It must therefore be of non-protein nature, and, if so, the pepsin formed from it by the action of acid can scarcely be a protein.

The physical properties of the substance are interesting. It was found to be slightly lævo-rotatory. We have already seen reason to suppose that enzymes are optically active. It is adsorbed by kieselguhr, alumina, and fibrin, but not by starch or sand. These facts serve to confirm the view of the "specific" nature of adsorption, since it can scarcely be held that pepsinogen has a greater chemical affinity for keiselguhr than it has for sand, although it would be expected to have more "affinity" for fibrin than for starch. Of course, the active surface of kieselguhr is greater than that of sand, and this may be the explanation of the above apparently specific adsorption, although other differences of surface must not be left out of account.

It is of interest to notice the close similarity between the enzyme and its zymogen, at all events in the present case. As Glaessner remarks, the conversion by very dilute mineral acids with such smoothness, rapidity, and completeness is only explicable on the assumption of a very simple chemical process.

It appears from the work of Hedin [1911] that, in the gastric mucous membrane, a substance is contained which inhibits the action of rennet. This substance is destroyed by acid, so that there is a possibility that the apparent zymogen may really consist in the simultaneous presence, in alkaline extracts, of the enzyme and the inhibitory substance. It is not clear, however, why alkali does not destroy the rennet, since it does so after activation by acid. Perhaps, the view taken is that it is in chemical combination with the inhibitory substance.

Zymogen of Lipase.

Yoshio Tanaka [1911] has shown that the lipase of the castor-oil bean is contained in the resting seed as a zymogen, and that acid, found by previous observers to be necessary for the activity of the extracts, is actually of use only for the purpose of converting the zymogen into enzyme. The lipase itself is most active in neutral medium. In a further paper [1912] this author investigates the optimal conditions for the conversion of the zymogen and gives the following useful method of preparing an active powder, which can be kept for a long time without losing its power: 100 grams of pressed castor seed, or after extraction to remove oil, are rubbed with 600-700 c.c. of decinormal acetic acid (or 500 c.c. of decinormal sulphuric acid) for thirty minutes at 30°-35°. The mixture is filtered, the residue washed thoroughly with water, in which the enzyme is insoluble, and dried at a temperature of 40°.

CHAPTER X.

OXIDATION-PROCESSES AND CERTAIN COMPLEX SYSTEMS.

INSTANCES have been given in the preceding pages where a system consisting only of enzyme and substrate undergoes no perceptible change. The addition of a third body is requisite in order that a reaction may take place. Such cases were those of lipase and zymase with their respective co-enzymes, as also pepsin and trypsin with acid and alkali respectively.

Oxidising Enzymes.

A different case of a complex system is that of the various enzymes concerned in the mechanism of oxidation. It will probably have been noticed that the enzyme-processes dealt with in any detail up to the present were all hydrolytic actions. The intimate mechanism of the oxidation enzymes is still comparatively little understood, and until the work of Bach and Chodat [1903], it was involved in much confusion. Although the point of view of these investigators may not as yet explain all the phenomena met with in this difficult subject, it is by far the most satisfactory hypothesis hitherto propounded.[1]

In the consideration of the subject there are three distinct sets of substances to be taken into account. These are :—

1. Organic peroxides and hydrogen peroxide.
2. Peroxidases.
3. Catalases.

The two last only are of enzyme nature. They both decompose hydrogen peroxide with formation of water and oxygen, but while the peroxidase separates oxygen in the active state, probably as atomatic oxygen, the catalase separates it as molecular or relatively inactive oxygen. The catalase must therefore attack two molecules of the peroxide simultaneously, since only one atom of oxygen is afforded by each molecule of peroxide. A second point of difference is that while catalase acts only on hydrogen peroxide, the peroxidases act on various organic peroxides; at any rate their general action can only be explained on this hypothesis, and Bach and Chodat have shown that ethyl-hydrogen peroxide is split by them.

[1] See the valuable summaries by Kastle [1910] and by Wolff [1910].

Catalase.

Now molecular oxygen is incapable of acting upon the greater number of substances, such as glucose, lactic acid, or uric acid, which are oxidised in the organism, so that, although catalase is very widespread, its function is somewhat problematic, except in the case of the green plant, as we shall see later. It is possible that hydrogen peroxide maybe produced as a bye-product of oxidations in the animal tissues; if so, it is necessary that it should be forthwith destroyed, owing to its toxic action on living protoplasm.

Oxidases.

A peroxidase alone, without the presence of a peroxide, is obviously of no use as an oxidising agent, so that the system of peroxide and peroxidase is the active combination. This system is, in fact, what is sometimes called an "oxidase". In order that the process of oxidation may be continuous, the peroxide component of the system must be capable of re-formation by taking up oxygen again. Accordingly we find that the oxidation of guaiacum by potatoes, for example, does not take place in the absence of oxygen.

An instructive experiment consists in the use of peroxidase, such as that to be obtained from the root of the horse-radish, instead of an inorganic catalyst in Fenton's reaction. If a mixture of lactic acid and hydrogen peroxide be taken, it will be found that oxidation proceeds with extreme slowness. A trace of ferrous sulphate will enormously accelerate the reaction. If the peroxidase of horse-radish be added instead of the iron salt, it is found that the reaction is accelerated in the same way. In other words, peroxidase is capable of taking the part of the iron salt in this reaction. This fact, in conjunction with that of the powerful effect of the addition of manganese or iron to various "oxidases," and the apparently universal occurrence of one or other of these bodies in the ash of these enzymes, suggests that the latter may be a means of rendering iron, manganese, or a similar body in an oxidisable system into an extremely active state. This suggestion remains as yet, however, in the region of pure hypothesis.

Dony-Henault [1908], however, describes the preparation of an artificial laccase by the precipitation, with alcohol, of a solution containing 10 grms. gum-arabic, 1 grm. manganese formate, and 0·4 grm. crystallised sodium bicarbonate in 50 c.c. water. The precipitate is redissolved in water and again precipitated by alcohol. This substance, no doubt a colloidal complex containing manganese hydroxide, has the properties of Bertrand's laccase and is of about the same degree of

activity. The function of the gum is probably that of a stable colloid which keeps the manganese hydroxide in a permanent highly divided, colloidal condition, so that a large active surface is maintained. The experiments are of great interest and importance, not only as the synthesis of an enzyme, but also as showing how an undoubted adsorption compound can be precipitated and redissolved without altering its properties. Repeated solution and reprecipitation of a uniform substance, indeed, cannot be held to be a proof of a definite chemical individual, so long as these processes are repeated under the same conditions.

Perrin has suggested [1905, p. 103] that an oxidase or rather peroxidase, essentially consists in the union of a hydrosol of ferric or manganic hydroxide, which is itself unstable, with a hydrophile colloid, which confers stability upon it. We have seen, in Fenton's reaction, how great is the activity of iron or manganese as a peroxidase. The experiments of Röhmann and Shmamine [1912] lead to similar conclusions.

We may summarise the results of the above considerations thus:—

Oxidation in the tissues is due to the presence therein, in the first place, of an organic substance, which is readily oxidisable by molecular oxygen. A part of the energy set free in this process is made use of to form from other molecules of the substance an unstable peroxide. Benzaldehyde undergoing oxidation in air is an instance of such a reaction. The analogous substance present in cells corresponds to what was originally called by Bach an "oxygenase," because it is by its means that molecular oxygen is activated. This activation is produced by the agency of an enzyme, peroxidase, which is a colloidal hydroxide of iron or manganese, kept active, or protected from precipitation or aggregation, by a stable or hydrophile colloid. This enzyme decomposes the peroxide with the liberation of "active" oxygen, with its powerful oxidising properties. We are still in ignorance as to what this form of oxygen is, but there seems to be much evidence to show that electrical forces play an important part; benzaldehyde, for example, in the process of oxidation, gives rise to the formation of gas ions, which cause condensation of a steam jet. It is taught by Ramsay that the active properties are manifested during the change of valency of oxygen, while it is in the process of gaining or losing electric charges. Looked at from another point of view, we may regard the active state of nascent substances as being the mode in which the energy of a reaction is enabled to be used for other chemical work before it has become degraded into heat.

Euler and Bolin [1908] find that laccase stands boiling, and that

salts of hydroxy-acids, in conjunction with manganese, have a powerful oxidising action on hydroquinone. Wolff [1908], also, shows that certain mineral salts can play the part of peroxidases.

At the same time, one cannot agree with the contention of Euler and Bolin that when the constitution of an enzyme is made out, it ceases to be an enzyme. Enzymes are merely a particular class of catalysts, considered for convenience apart, owing to the fact that they are produced by living organisms and are, for the most part, of unknown chemical constitution.

In the experiments of Herzog and Meier [1911] there seems to be relationship between the amount of peroxidase added and the amount of oxidation brought about by a given quantity of hydrogen peroxide. This is due to the destructive action of the peroxide on the enzyme, since the latter was found to be absent at the end of the reaction, when small amounts were added. It is readily to be understood why the amount of oxidation was also proportional to the concentration of the hydrogen peroxide, since the oxygen used in the reaction is afforded by it.

The chief difficulty in the universal application of the Bach-Chodat theory lies in the very specific nature of many oxidases. Laccase was shown by Bertrand [1896] to act upon hydroquinone or pyrogallol with great facility, but not at all on resorcinol or phloroglucinol. Moreover, it does not act upon tyrosine, which is readily oxidised by another enzyme, "tyrosinase". A satisfactory explanation of these facts is wanting. It would be expected that active oxygen liberated by the agency of a peroxidase would be able to effect oxidations indiscriminately, and the experiment on lactic acid and hydrogen peroxide cited above seems to indicate that the specificity lies in the peroxide component of the oxidase system. If this be so, it is possible that there is some intimate relationship necessary between the structure of the peroxide and the substrate in order that close connection may be possible so that the active oxygen may enter into immediate union with the latter.

With regard to the oxidising powers of various tissues, the work of Vernon [1911] on the indophenol oxidase may be consulted. Those tissues which have the greatest oxidising action are those which Ehrlich found to have the highest degree of "oxygen saturation"; so that it appears that the so-called "intramolecular" oxygen, when present, is stored as organic peroxide of some kind, not as a constituent of a "biogen" or protoplasmic molecule. As peroxide, acting as carrier of active oxygen, it would only be present at any given moment in very minute amount.

Reductases.

Until recent times it was doubtful whether any part was played by enzymes in the phenomena of reduction shown by living cells. The observations of Schardinger [1909] that, although fresh milk alone has no action on methylene blue, etc., it reduces such dye-stuffs to leucobases if formic or acetic aldehyde is added, suggested the presence of an enzyme. This view was further strengthened by the fact that the result is absent when the milk has been boiled. On the other hand, if bacteria develop, substances are produced which reduce methylene blue without the necessity of an aldehyde. There is an obvious similarity between these facts and those of the peroxide-peroxidase system, as Bach [1911] points out. In fact, this observer has been able, from extracts of calf's liver, to precipitate by alcohol an enzyme which gives the reactions of Schardinger; *i.e.*, it reduces methylene blue, but only in the presence of aldehyde. Other reductions, such as that of nitrates to nitrites, are also brought about by its agency.

Now, just as active or nascent oxygen is the most effective oxidising agent, active or nascent hydrogen is the most effective reducing agent. This being so, we are led to look for evidence of compounds similar to the peroxides, perhydrides, as they may be called. Taking the quadrivalence of oxygen as an established fact, we see the probability of an oxygen perhydride, H_4O, corresponding to the hydrogen peroxide, $H_2 = O = O$. Although no definite organic compound of similar constitution to H_4O has been as yet isolated, it is an attractive hypothesis to work with to assume that, as peroxidase sets free nascent oxygen from peroxides, Schardinger's enzyme, which might be called a perhydridase, sets free nascent hydrogen from organic perhydrides.

As Bach points out [1911, p. 41], if this point of view be accepted, we can divide enzymes into three classes :—

1. Those which act on *hydrogenated water* (hydrogen suboxide, or oxygen perhydride) and are concerned with reduction.

2. Those which act on *water* itself and are concerned with hydrolysis. Most of the ordinary enzymes belong to this class.

3. Those which act on *oxygenated water* (hydrogen peroxide) and are concerned with oxidation processes.

The " reductases " or hydrogenases are probably, like the oxidases, compound systems of perhydridases with unstable organic perhydrides.

In all phenomena of oxidation or reduction, as it is unnecessary

to remind the reader, when one substance is reduced another is at the same time oxidised. The difference in the two cases before us is in the means by which the reaction is brought about, whether it is effected by the activation of oxygen or of hydrogen. In this connection, the work of Bredig and Sommer [1910] on the action of colloidal platinum as a reducing catalyst and the relation of the phenomena to the coupled reactions, to be described in the following section of this chapter, may be consulted with advantage.

Coupled Reactions.

There is an interesting class of chemical reactions called by Ostwald [1900] "coupled reactions," which it seems probable will have to be taken into account in oxidation, and perhaps also in other processes in the organism. An instance of such a reaction is the following: When a solution of sodium arsenite is shaken with air no oxidation takes place; sodium sulphite similarly treated is rapidly oxidised; if a mixture of the two be shaken with air *both* salts are oxidised. In some way or other oxygen is activated in the process. It is convenient to give names to reactions in which a slow reaction is accelerated by the existence of a simultaneous rapid reaction between one of the reacting bodies of the former reaction and a third body. The rapid reaction is called the "primary reaction," and that which is induced by the presence of the former is called the "secondary reaction". The body common to both is called the "actor"; the second substance in the primary reaction is the "inductor"; and the corresponding body in the secondary reaction is the "acceptor". In the example given, oxygen is the actor, sodium sulphite the inductor, and sodium arsenite the acceptor. Certain catalytic reactions already referred to may be described from this point of view; the catalysis by ferrous sulphate of the oxidation of hydriodic acid by hydrogen peroxide is one of these. In this process, the reaction $FeSO_4 + H_2O_2$ is the primary reaction, $H_2O_2 + HI$ is the secondary reaction, H_2O_2 is the actor, $FeSO_4$ the inductor, and HI is the acceptor. For further information on this question, as yet imperfectly worked out, the reader is referred to Mellor's *Chemical Statics and Dynamics*.

The Chlorophyll Function.

In connection with the properties of catalase, the researches of Usher and Priestley [1906, 1 and 2, 1911] on the chlorophyll function are of interest. According to these observers, this system consists of three partners—the protoplasm of the chloroplast, the chlorophyll itself, and a catalase. By means of the pigment, acting as both optical and chemical sensitiser, light-energy is employed to cause reaction between carbon dioxide and water in such a manner that formaldehyde and hydrogen peroxide are formed. Both of these bodies are toxic, and, if allowed to accumulate, the reaction soon comes to an end. The formaldehyde is, however, rapidly polymerised by the protoplasm of the chloroplast and the hydrogen peroxide is split into oxygen and water by the catalase. We see thus why the reaction as a whole does not occur in non-living preparations or extracts of green leaves. Formaldehyde is indeed produced in the presence of chloroform on exposure to light of appropriate arrangements of the pigment, but since no polymerisation occurs, the chlorophyll is destroyed by it and the reaction comes to an end. Production of hydrogen peroxide and formaldehyde also takes place in leaves killed by boiling, and in this case, since the enzyme, catalase, is destroyed, as well as the protoplasm, the hydrogen peroxide also contributes to the destruction of the chlorophyll. It is well to mention that some doubt has recently been thrown on these results by Ewart [1908], but, as far as the production of formaldehyde by solution of chlorophyll is concerned, they have been confirmed by Schryver [1910]. The objections of Ewart are answered by Usher and Priestley [1911] in a third paper, in which further evidence is brought forward in support of the view that the light reaction is as described above.

In this reaction, light does not act as a catalyst, since the energy of the sun's rays is stored up in the products.

In a catalytic process, as has been frequently explained in the preceding pages, there is no change in the energy-content of the system, whereas, in photo-chemical reactions, such changes take place. There is a large body of evidence to show that light acts in a similar manner to that of a rapidly alternating electric field, in fact a species of electrolysis occurs. This is not surprising when we remember the Maxwell electromagnetic theory of light. Walther Loeb [1905] has obtained formaldehyde and hydrogen peroxide from moist carbon dioxide by the action of the rapidly alternating silent discharge.

The Coagulation of Blood.

The factors concerned in the coagulation of the blood form another extraordinarily complex system. According to Morawitz [1903, 1905], there exists in circulating blood a body, thrombogen, which can be converted by a thrombokinase, present in all tissues,[1] including the formed elements of the blood, into a precursor of the enzyme which acts upon fibrinogen to form fibrin. This precursor, or prothrombase, is changed into the active thrombase by calcium ions. According to Nolf [1906-7, 1908], the process consists essentially in the interaction of three colloidal proteins in the presence of calcium ions. These proteins are thrombogen, thrombozyme, and fibrinogen. Both fibrin and thrombase consist of all three of these colloids, but in different proportion; fibrin contains fibrinogen in excess, a circumstance which accounts for its insolubility. Thrombozyme is a proteolytic enzyme, the process of coagulation being the first stage of its action, while the well-known fibrinolysis is the second stage. This brief statement gives but a mere abstract of this important work which is, in the main, a confirmation and extension of that of Wooldridge. For further details the original papers must be consulted.

The work of Rettger on this question [1909] is also of much importance. He shows that "fibrin-ferment" is not destroyed by heat, and that it enters quantitatively into the products of the reaction. It is therefore not an enzyme. Further discussion of the subject does not, for this reason, fall within the scope of this monograph, notwithstanding its intrinsic importance.

Alcoholic Fermentation.

Finally, under the heading of this chapter may be mentioned those fairly numerous cases where more than one enzyme is requisite to effect a particular change. Here the enzymes act one after the other, and the products of the one become the substrate of the next. According to Buchner and Meisenheimer [1910], the system responsible for the alcoholic fermentation of sugar is a complex one. Contrary, however, to their original view that lactic acid is the intermediate product, supposed to be attacked by a second enzyme, "lactacidase," they now find that dihydroxy-acetone is, in all probability, the body in question. Lactic acid, in fact, is not fermented by yeast. The question cannot be regarded as being finally decided at present. In the animal organism, it appears that glyceric aldehyde is the most im-

[1] With respect to the properties of tissue-extracts, the paper of Pekelharing [1908] should be consulted.

portant intermediate product in the breakdown of sugars, although it is not so readily fermented by yeast-juice as dihydroxy-acetone is. For further discussion see the monograph on *Alcoholic Fermentation* by Harden in this series.

Incidentally referred to in the work of Buchner and Meisenheimer there is an interesting point with regard to the theory of catalysis, as discussed on p. 7 above. Glucose kept for five years in a sealed tube was found not to have given rise to any production either of carbon dioxide or of alcohol.

Hydrolysis of Gentianose.

Another interesting case is that of the enzymatic hydrolysis of gentianose, as investigated by Bourquelot and Hérissey [1902]. This sugar is a tri-hexose, consisting of two molecules of glucose and one of fructose. If it be subjected to the action of invertase the fructose is split off, leaving the two molecules of glucose still united as a bi-hexose. Emulsin, or rather the mixture of enzymes obtained from the bitter-almond, is able now to split this bi-hexose into its components. The importance of this case is that if a mixture of emulsin and invertase had been added at once the gentianose would have been completely hydrolysed; the juice of *Aspergillus* appears to contain such a mixture, which might have been mistaken for a single enzyme but for the previous experiments. It has recently been shown by Armstrong and Horton [1908] that the "emulsin" of the almond contains three distinct enzymes. These are, a lactase, a β-glucase, which causes the hydrolysis of β-glucosides, such as salicin, while the third is an enzyme which separates glucose from amygdalin, leaving Fischer's amygdonitrile-glucoside.

Hydrolysis of Raffinose.

Raffinose, a tri-hexose consisting of glucose, fructose, and galactose, as shown by Fischer and Lindner [1895], requires the action of a series of enzymes to effect its complete hydrolysis. According to Armstrong and Glover [1908], when hydrolysed by acids or invertase, it is resolved into fructose and melibiose, while the lactase of "emulsin" converts it into cane-sugar and galactose, as first shown by Neuberg [1907].

Hydrolysis of Starch.

For the conversion of starch into glucose, as we have already seen, three enzymes have been postulated. Amylase transforms it into dextrins, which are then converted by the dextrinase of Duclaux into maltose, which is finally changed to glucose by maltase.

The results of Maquenne [1906] and of Wohl and Glimm [1910], already referred to, seem to show that this assumption is probably unnecessary.

Proteoclastic Systems.

The same kind of phenomena are observed in the series of proteoclastic enzymes met with by the food as it traverses the alimentary canal. Erepsin acts upon the products of the action of trypsin, while this latter enzyme itself acts upon the products of peptic digestion.

The Arginase System.

Another system which may be added to the preceding is that which may be called the arginase system. An enzyme was prepared by Kossel and Dakin [1904] from the liver, which has the property of converting arginine into urea and diamino-valerianic acid. Now arginine is found in considerable amount in germinating seeds, especially those containing much protein store, such as those of the lupin, as shown by Schulze and Winterstein. It occurs in this place as a product of protein hydrolysis. Urea, in its turn, is hydrolysed by an enzyme, urease, into ammonia, and urease has been found in the seeds of another leguminous plant, the Soy bean, by Takeuchi [1909]. Although, so far as I am aware, urease has not as yet been found in conjunction with arginase, it seems very likely that, when looked for, they will be discovered together, especially since ammonia is such an important food-stuff for the growing plant.

GENERAL CONCLUSIONS.

THE living organism is enabled by the use of enzymes to bring about, under ordinary conditions of temperature and moderate concentrations of acid or alkali, many chemical reactions which would otherwise require a high temperature or powerful reagents.

A careful study of these enzymes shows that they obey the usual laws of catalytic phenomena. Certain deviations from the behaviour of most inorganic catalysts are found to depend upon the colloidal nature of enzymes, so that the reactions take place in a heterogeneous system and the various phenomena depending upon surface action come markedly into play.

As they are sensitive to heat and more or less rapidly destroyed by it, they show the phenomenon of a so-called *optimum* temperature. This destruction by heat is, in all probability, due to their organic colloidal nature.

So far as we know, the reactions catalysed by enzymes are reversible in nature, but since, as investigated *in vitro*, they usually take place in presence of excess of water, the equilibrium-position is very near the stage of complete hydrolysis. Owing to this reversible character of the reactions, it follows that enzymes have synthetic action.

In the organism, mechanisms appear to be present which are able to diminish to a large extent the effective concentration of water, so as to favour the synthetic reaction, when required.

Reasons are given for the belief that the "compound" of enzyme and substrate, generally regarded as the preliminary to action, is of the nature of a colloidal adsorption-compound; so that the action of enzymes in general must be regarded as exerted by their surface. By surface condensation the reacting constituents are brought into intimate contact and reaction accelerated by mass-action. Whether chemical combination between enzyme and substrate occurs in any stage of the process is not yet decided. Direct experimental proof exists that enzymes act by their surfaces in liquids in which they are completely insoluble.

The existence of relations of this kind explains the exponential

form of the law correlating the concentration of enzyme with its activity.

Autocatalysis, positive and negative, plays a considerable part in the changes of activity of an enzyme during the course of its action. These changes of "activity" are the main factors in the deviations of the form of the equation for the velocity of reaction from the simple unimolecular formula, when the reaction, as is usually the case, takes place in presence of excess of water.

SUPPLEMENTARY NOTES.

NOTE A.

IN illustration of the great activity of the organic agents or enzymes which effect these changes, as compared with inorganic hydrolytic agents, the following experiment of Frankland Armstrong [1904, 3, p. 533] may be quoted: A preparation of lactase was found to hydrolyse about one-fourth of the milk-sugar contained in a 5 per cent. solution in one hour at 35°, whereas a twice normal hydrochloric acid required, at the same temperature, about five weeks to effect the same amount of hydrolysis.

NOTE B.

It should be mentioned that this formation of amino-acids (Lawrow [1898-99, p. 516]) is regarded by some as being due to the presence of autolytic enzyme in ordinary pepsin preparations. This does not seem very probable, however, in view of the difficulty of obtaining solutions of autolytic enzymes, owing to their intracellular nature. Hirayama [1910] shows that natural gastric juice is capable of producing a high degree of hydrolysis of proteins under certain conditions, such action as is exerted by Hedin's spleen protease. It will be remembered that Fischer and Abderhalden [1905, p. 55] found that the pancreatin "Rhenania," which appears to be dried pancreas, was able to hydrolyse leucyl-alanine to some degree, whereas fresh pancreatic juice was unable to do so. Dried pancreas would probably contain a small amount of autolytic enzyme.

NOTE C.

The following remarks by van't Hoff [1898, p. 12] will be read with interest: "The theory of chemical equilibrium may also find its application here (*i.e.*, in organic chemistry) and indeed has already done so; on account of the great variety of compounds and the inertia of reaction, however, appropriate choice of material is not easy. It is, perhaps, on this account, of some value to direct attention to the

extremely noteworthy ferment- or enzyme-actions, which have shown themselves admirable for this purpose, as recent investigations demonstrate. On the one hand, Fischer [1894] found that, under the action of ferments, organic changes are directed into determinate paths, a fact which completely excludes complication by variety of forms. On the other hand, the recent researches of Tammann [1889, 1892, 1895], Duclaux [1898] and especially of Hill [1898] cannot be explained without the introduction of considerations of equilibrium. It was pointed out by Tammann that, under the action of emulsin, amygdalin is only partially split, and that this hydrolysis proceeds further if the products are removed. Perhaps if he had added a further amount of products of hydrolysis, he might have succeeded in synthesising amygdalin. Duclaux put forward transformation formulæ, which again suggest the attainment of an equilibrium, and Hill seems to have effected the synthesis of maltose from glucose by means of a yeast enzyme. Unless a ferment undergoes alteration of some kind during its period of activity, it follows, on theoretical grounds, that a condition of equilibrium and not one of total change must be brought about, and that therefore the opposite reaction must be induced. We are indeed justified in asking the question, whether (by application of the theory of equilibrium), under the influence of zymase and by exceeding a certain limiting opposing pressure of carbon dioxide, glucose might not be formed from alcohol and carbon dioxide, and moreover whether trypsin may not be able, under conditions prescribed by the theory of equilibrium, to form protein from the products of the hydrolysis which it brings about under other conditions." [1]

NOTE D.

Enzymes do not differ from chemical catalysts of known composition in their behaviour towards optical isomers, as has been shown recently by Bredig and Fajans [1898]. The d- and l-camphor-carboxylic acids in solution in acetophenone slowly decompose with evolution of CO_2. This reaction is catalysed by bases. When an optically inactive base is used, both acids are decomposed at equal rates, but if an optically active base, such as nicotine, be used, it is found that the d-acid is acted upon at a rate which is 17 per cent. faster than that at which the other acid is decomposed. As ordinary nicotine is lævo-rotatory, it would be of much interest to know whether

[1] See also the papers on "Synthetic Ferment Action" by the same author in *Sitzungsber. d. Kgl. Pr. Akad.*, 1909, 42, 1065-1076, and 48, 963-970.

the dextro-rotatory nicotine would decompose the l-acid faster than the d-acid. See also the full paper by Fajans [1910].

Asymmetric synthesis of benzaldehyde-cyanhydrol has been obtained by Bredig and Fiske [1912]. When an optically active base is used as catalyst, the particular optical isomer formed depends on the sign of the rotation of the base.

NOTE E.

On the Specificity of Enzymes.

The usually accepted doctrine of the extreme specificity of enzymes has been tacitly accepted in the text of this monograph. But there are many facts claiming attention, which suggest that caution is necessary before unconditional acceptance of this view.

It is, at the present time, a matter of custom to postulate the existence of a new enzyme whenever some substrate, previously unknown to be attacked by any enzyme, is found to be so attacked, either by a well-known preparation or by a newly discovered one. It is stated, for instance, that "emulsin" consists of, at least, four distinct enzymes—benzcyanase, amygdalase, β-glucase, and gluco-lactase.

More work is requisite in order to decide whether these various actions are really due to distinct enzymes or are merely differences in the action of one and the same enzyme, according to varying conditions in the reacting system. If the enzymes are really distinct, it ought to be possible to separate them. In this case, it is necessary that the enzymes supposed to be different be tested in precisely similar conditions; this requisite was not fulfilled in the work of Rosenthaler on "synthetic" emulsin, so that the results are of little value as regards the proof of the existence of two distinct enzymes.

When a set of varying effects is obtained by the action of an enzyme preparation on different glucosides, as in the experiments of Armstrong and Horton [1913] with emulsin from clover grown on different soils, I venture to suggest that it might repay investigation to find out whether the action of one single enzyme cannot be slightly modified by the presence of other substances in the reacting system. The giving of a new name is apt to obscure the close similarity of the supposed new enzyme to others already known.

It may be objected that the doctrine of "lock and key" is unduly neglected, unless the doctrine of specificity is completely acknowledged. It appears, however, that Emil Fischer himself did not intend that his simile should be applied in the extreme way that his

successors have applied it. His own experiments were, in fact, not continued for a sufficiently long time to decide whether a particular enzyme might not slowly attack a substrate differing from that on which it is particularly active. Moreover, it must not be forgotten that there are such things as "master-keys" which are capable of opening several different locks. Such a possibility is admitted even by the Armstrongs [1907, p. 365].

A statement made by Emil Fischer [1895, p. 853], of the Collected Papers on "Kohlenhydrate") is to the point here. After referring to the fact that different enzymes appear to have different degrees of activity on the same substrate, Fischer proceeds, "we are at present in the dark as to the cause of these differences. It is possible that emulsin and similar substances are different enzymes, as has been assumed in the case of diastase. But one can also picture to oneself the same chemical molecule as capable of different enzymic activities, according as it enters into reaction with one or other group of atoms. It is finally also not unthinkable that the important group in one enzyme may have a greater activity than it has in a related enzyme, on account of small differences of configuration."

The subject needs attacking rather from the dynamic than from the static point of view, rates of reaction need more investigation than fitting of locks and keys. If we accept Ostwald's definition of catalysis, the simile is not a very appropriate one, since locks do not open themselves, however long a time be allowed for the process to take place. Much of the recent work on the nature of enzyme action tends to show that the point of view of pure structural chemistry gives very little help in the difficult problem, although, no doubt, there are certain questions which await attack from this standpoint. Such a problem is that of the nature of the intermediate chemical compound between enzyme and substrate, which is supposed to be formed and afterwards decomposed; if, indeed, such a compound actually exists.

There is, undoubtedly, much experimental difficulty in deciding the question. When a purified preparation is found to act upon, say maltose and amygdalin, but upon the latter at a slower rate than upon the former, the adherents of extreme specificity may reasonably object that a small amount of "emulsin" is still present. At the same time, the difficulties can probably be overcome.

It is shown by Fajans [1910] in detail how much more simply the various experimental data as to the action of the different enzymes acting on optical isomers, including synthetic action, can be explained by the hypothesis of both isomers being hydrolysed by the same enzyme

but at different rates. This view does not, of course, state that maltase and emulsin, for example, are one and the same enzyme. It states that maltase acts upon both α- and β-glucosides, but upon the latter at a slower rate; emulsin, on the other hand, acts more rapidly upon the β-glucoside. It does, not, indeed, exclude the possibility of their being the same body, although at present there is no direct evidence for this. For the details of the mathematical treatment of the theory, the reader is referred to the original paper. In this place a few facts to which Fajans calls attention may be mentioned.

It will be remembered that in the work of Dakin on lipase [1907], of which an account has been given in the text, the two optically isomeric mandelic esters were found to be hydrolysed at different rates, but were finally both equally attacked, the system in equilibrium being optically inactive. As regards lipase, then, the hypothesis as stated above is found to hold. Although the difference in rate of action in these cases is much less than it is necessary to assume for such enzymes as maltase and emulsin, it is found that in actual instances there are all kinds of differences of degree in this respect (see Fajans, p. 77). Similar facts are known in the hydrolysis of polypeptides. Here the curious preferences shown by tryptic enzymes may be noted; these are not satisfactorily explained by assuming a close chemical relationship of enzyme and substrate, and seem to suggest rather differences of a physical nature.

The rule of the one optical isomer being the only one attacked by the living organism is by no means universal. Bacteria have been shown by Neuberg [1909], and moulds by Pringsheim [1910] to be able to utilise *both* components of racemic amino-acids. The oxidases of fungi act on both isomers of amino-acids, but at different rates. The different rates of action of extracts of *Aspergillus* on α and β-glucosides as found by Dox and Neidig [1912] may also be referred to. Kondo [1912] found that the liver is capable of synthesising amino-acids from the corresponding ketonic acids and ammonia, even when the acids in question are not such as occur in the proteins of the organism. Since the one isomer is not of natural occurrence, but notwithstanding this it is attacked, it follows that, if a special enzyme be required, these organisms have produced in the course of their evolution an agent which has never had a chance of exerting its activity until the organisms fell into the hands of the physiologist.

One may recall the apparent production of enzymes where they are never made use of, or, if given the opportunity of acting, would produce toxic substances, for example, emulsin in the alimentary

canal of vertebrates (Brissemart and Combes [1906]), rennet in plants (if it be supposed that it is a different enzyme from papain), lactase in almonds, and so on. There are two alternative explanations of these facts. If the actions mentioned are due to the enzymes named, it is possible that such enzymes might be produced as bye-products of metabolism, independently of use being made of them. The other alternative is that the action is due to some enzyme which does other things as well. In either case, we must give up specificity, except in a very wide sense.

Moreover, it is suggestive in the present connection that recent investigations are showing, one after another, that in various situations where either maltase or emulsin was supposed to exist alone, the activity supposed to belong only to the opposite enzyme also makes itself evident. I refer to such cases as the yeast-cell, the mucous membrane of the alimentary canal (Brissemart and Combes [1906]), etc.

To turn for a moment to synthetic action, Fajans points out that, if the velocity constants of the action of any particular enzyme on opposite isomers are not identical, the result in equilibrium may be preponderance of the one or the other ; we then have the asymmetrical synthesis of Rosenthaler [1908]. Fajans also shows how the results of Rosenthaler can be explained on the theory of catalysis by optically active catalysts, without assuming the existence of any special enzyme. In other words, it is not necessary that the relative synthetic effect should be the same as the relative hydrolytic effect, when optical isomers are concerned. If they are not the same, an optically active body will be synthesised. If maltase, for example, hydrolyses maltose more quickly than it does isomaltose, then, when synthetic action occurs, isomaltose may well be produced at a greater rate than maltose, so that in equilibrium isomaltose will preponderate; the same result will happen even if both isomers are produced at equal rates, if one of them is hydrolysed more quickly than the other. Rosenthaler's work on the synthesis of optically active benzaldehyde-cyanhydrol by emulsin showed that both isomers were finally present in the system in equal amount.

I am unable to understand the grounds on which Armstrong and Horton [1912] base their objection to the experiments of Fajans as having no connection with enzymes, *viz.*, that the optically active base does not function as a catalyst. It is shown in the paper of Fajans [1910, p. 61] that the base does not enter into the constituents of the final equilibrium and that it does not act in stoichiometrical proportion to the substrate. The exact mechanism by which the action is exerted does not concern the question ; catalytic actions may be exer-

cised through the formation of intermediate compounds. Further, even if we admit that oxidases themselves are not enzymes, an enzyme is a necessary component of the oxidising system. The admission on the part of the authors named that amino-acids are readily racemised seems to me to deprive the doctrine of specificity of a large part of its experimental basis. Why should not glucosides and sugars also be racemised in the process of synthesis? As to lipase, surely it has some connection with optical activity when it hydrolyses one optical isomer faster than the other, as in Dakin's experiments.

Certain considerations force themselves on one's attention on reading the paper by Armstrong and Horton [1910] on the actions of the enzymes of the emulsin type.

The initial action of "emulsin" on amygdalin is the same as that of maltase, *viz.*, the separation of mandelic nitrile glucoside, an action also shown by sulphuric acid under certain conditions. This does not seem then to be a very specific form of activity.

The authors themselves find difficulties in regarding emulsin as "compatible" with glucose alone, and apparently admit the possibility of different results from the same enzyme under different conditions (see p. 351 of the paper). They lay stress on the importance of experiments being done under "molecularly" comparable conditions when they are discussing Dunstan's view that phaseo-lunatin is an α-glucoside, while they themselves hold that it is a β-glucoside.

Although it is regarded as a β-glucoside, emulsin has but little action on it; an enzyme "phaseo-lunatase," prepared from the same beans as the glucoside itself, is much more effective. Now this enzyme has scarcely any effect on amygdalin, although it acts upon mandelic nitrile glucoside. The result is explained by the statement that it is destitute of "amygdalase". But if it hydrolyses mandelic nitrile glucoside, why does it not split amygdalin at the same place, *viz.*, into a disaccharide and benzaldehyde-cyanhydrin?[1] Another hypothesis is necessary, that one glucose must first be removed before phaseo-lunatase can act. β-glucase is finally subdivided into several specific enzymes.

When one reads that an enzyme attacking β-glucosides is a β-glucase, that a body which is attacked by it is a β-glucoside, and a body unattacked is not a body of this kind, one may, I think, be excused the misgiving that the whole is rather like an argument in a circle.

[1] This appears to be done by an enzyme from the snail (Giaja, *Comptes rendus* [1910, 150, 793-796]).

It does not seem altogether impossible that enzymes may, under special conditions, change the rotation of an optically active body, since racemisation is not difficult by inorganic catalysts and enzymes, as we have seen, are more powerful than these.

Finally, if the differential action of an enzyme preparation on optical isomers is a matter of relative rate, heating to a temperature at which it appears to lose its effect on one or other of the isomers may be merely reducing the less powerful action to one inappreciable within a reasonable time of observation. A similar remark may be made with respect to the supposed fractional separation by precipitating reagents, and, in fact, I have been able to show experimentally [1913, p 254] that this is the explanation of the results of Rosenthaler with regard to the preparation of emulsins which act in either a hydrolysing or synthesising manner only.

Recent work has brought about a certain degree of simplification in the case of two enzymes, rennet and lipase. Pavloff and Parastschuk [1904] propounded the view, based on experimental evidence, that rennet action is merely an aspect of the action of pepsin under special conditions, *viz.*, neutral reaction, and with the calcium salt of caseinogen as substrate. Subsequent investigators have confirmed this, although it is not universally accepted (see Oppenheimer [1909, pp. 287-92]). Miss Porter [1911] regards the presence, in rennet preparations, of an antipeptic substance which does not inhibit the rennet action, as proving the independence of the two enzymes. It is to be remembered that the clotting of milk is a colloidal reaction and probably has nothing to do with the genuine enzyme activity of pepsin. This fact puts great difficulty in the way of deciding the question. The want of parallelism between clotting and peptic activity does not seem to me to militate against the view of their being due to the same substance. It has been shown that the action of rennet involves no hydrolysis (Couvreur [1910]). Further evidence on the question may be found in the papers by Van Dam [1912], Funk and Niemann [1910], and Rakoczy [1910]. It is held by Oppenheimer that different enzymes are responsible for the hydrolysis of simple esters and of the higher fats [1909, pp. 5 and 7], even monobutyrin and amyl salicylate require separate enzymes. One of the main reasons given for this view is the different action of bile-salts in the various cases. Now Terroine [1910, 3] showed conclusively that, in order to compare properly these actions, esters of the same acid must be taken. He took, therefore, triacetin, as glycerol ester, and the acetic esters of methyl, ethyl, propyl, and amyl. In all these cases the *optimum* concentration of bile-salts was precisely the same. He concluded that the same enzyme is con-

cerned. Naturally, when the system is heterogeneous, as when the higher glycerides are used, the conditions are not the same as in the homogeneous systems, and it would be surprising if the optimal concentration were the same. Wohl and Glimm [1910] give reasons for regarding the separation of amylase into two enzymes by Fraenkel and Hamburg [1906] as being an unnecessary assumption, since the facts can be explained by taking account of the different colloidal state of the enzyme in the two cases. The activity of an enzyme varies greatly with the colloidal state, both of itself and of its substrate.

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